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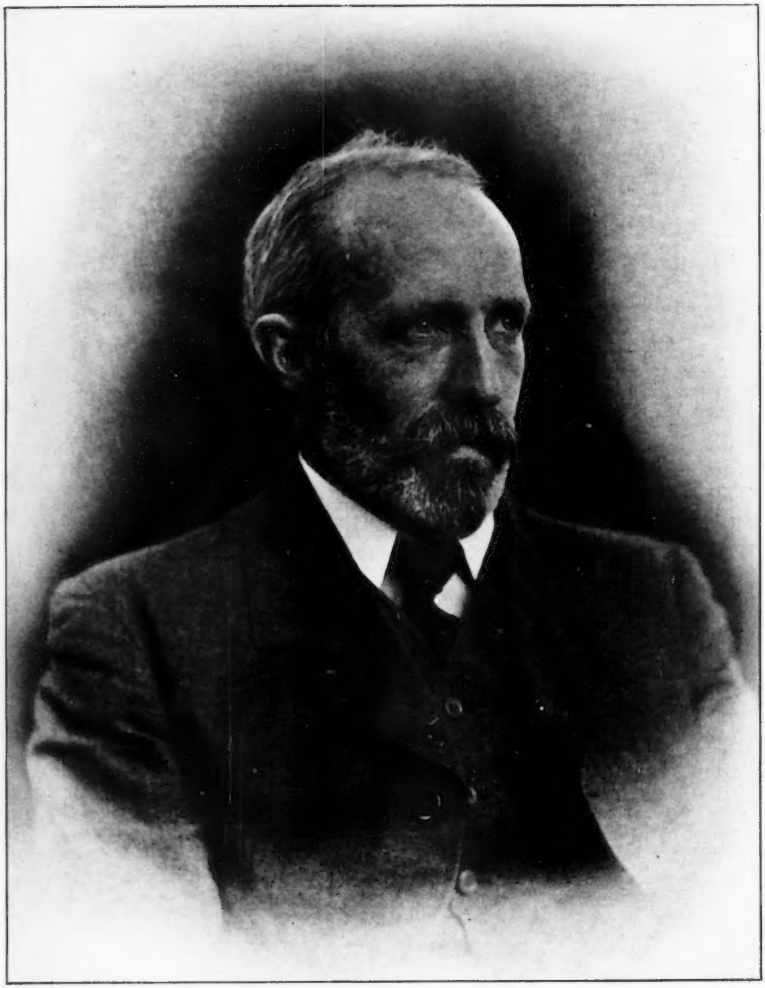

The late MR. ARTHUR W. BACOT,
Entomologist to the Lister Institute, London.

THE BRITISH JOURNAL

OF

EXPERIMENTAL PATHOLOGY

IN MEMORIAM.

ARTHUR W. BACOT, F.E.S.,

Entomologist to the Lister Institute, London.

Born April 28th, 1866, *Died April 12th*, 1922.

By the death of Arthur Bacot, Entomologist to the Lister Institute, from typhus fever contracted in the course of experimental research into the ætiology of this disease in Cairo, medical entomology loses an investigator whose unique attainments had gained him world-wide repute. It was only in January of this year that Bacot and his colleague Arkwright proceeded to Cairo at the request of the Egyptian Government to continue researches into the nature and life-history of the typhus virus which in London were not fully satisfied by the employment of passage virus only. So far as technical methods went, the transference to Cairo meant to them simply a change of laboratory. They were accommodated at the Hygienic Institute in rooms placed at their disposal by the Director, Dr. Charles Todd, an old colleague of both. Precisely how Bacot, and shortly afterwards Arkwright, now happily convalescent, became infected, will perhaps never be known. In the course of their work they had found, in contrast to their own previous experience in London (see Atkin and Bacot, *Brit. J. Exper. Pathol.*, not yet published) and to that of Rocha-Lima, but in harmony with statements of Nicolle, that the excreta of typhus-infected lice were capable of conveying the disease to guinea-pigs, and it is just possible that infection arose from this source, especially as their work involved much handling of louse-boxes charged with virulent fæcal material. Both experts, however, knew well the risks they ran, and while providing against accidents so far as humanly possible, were not deterred from the pursuit of knowledge by the chance of some unlooked-for avenue of infection. To the long list of workers whose labours to solve the mystery of typhus fever have claimed the last penalty must now be added the name of Arthur Bacot.

At his death Bacot had all but completed his 56th year, and it was only 11 years ago that the opportunity came to him to bring his great knowledge of insect lore and bionomics—the fruit of a boyhood's hobby but the scientific passion of maturer years—to bear on the elucidation of vital problems of epidemiology. He was educated at the Birkbeck School, on leaving which he became a clerk in a city office, and there he remained till he was 45 years of

age. In his hours of leisure, however, he turned to his insect studies, and much of his early work in entomology was concerned with breeding experiments in Lepidoptera with special reference to analysis of Mendelian phenomena. Bacot was most successful as a breeder of insects for statistical analysis on Mendelian lines, and he was equally successful when at a later period in his career he undertook the study of blood-sucking insects in connection with human disease. At practically all times or at shortest notice Bacot could supply for his own experiments or those of others, lice, bugs or mosquitoes fed on his own person. Bacot rapidly became a lepidopterist of repute, and many of his contributions to entomology between the years 1890–1910 will be found in various entomological journals such as *The Entomologist's Record* and the *Proceedings of the Entomological Society of London*, of which he became a Fellow in 1901. In 1910 Bacot was requested by the Plague Research Commission to undertake a study of the bionomics of fleas, a subject on which the precise information available was but meagre. Further knowledge was indispensable to the elucidation of the *rôle* played by the rat flea in the transmission of plague from rat to man. Bacot undertook the work with enthusiasm, and by the provision of an assistant it was made possible for him to organise and supervise the multifarious experiments connected with the problem without totally severing his connection with the office stool. Within 18 months the ground had been largely covered, and the result was a compendium of knowledge on the life-history and bionomics of rat fleas which for all time must form the hunting ground of workers in this sphere. The experiments in connection with this work were carried out at Loughton, Essex, where Bacot lived, and the collated results are embodied in a lengthy monograph contributed to the *Journal of Hygiene*.

This research and its outcome so completely displayed Bacot's talents for undertaking and bringing to a conclusion a piece of systematic work under rigidly controlled conditions, that it became obvious that his attainments must be given scope for their fullest development by the opportunity of uninterrupted devotion to the medical aspects of entomology. He was consequently invited, in 1911, by the Governing Body of the Lister Institute, to accept the position of Entomologist to the Institute—a new post specially created for him. Henceforward he could devote himself solely to the development of his specialty and its application to current medical and epidemiological problems. To take an effective part in research requiring the services of variously trained experts, necessitates a very careful dovetailing of the various units concerned. To fit himself for such collaborative work Bacot was not long in familiarising himself with technical procedures hitherto new to him, such as sectioning and staining of insect tissues, and such was his handiness that he became most adept in this difficult art. A working knowledge of bacteriological technique also did not come amiss to him, so that in a remarkably short space of time he was able to undertake some complete problem unassisted or take an effective share in the problem of a team.

The exact mechanism of transmission of plague from rat to rat or rat to man by means of the rat flea was not clear.

The matter was clarified by a fascinating piece of work carried out by Bacot in conjunction with C. J. Martin. It was found that in the stomach of

the flea (*Xenopsylla cheopis* and *Ceratophyllus fasciatus*) which has sucked blood containing plague bacilli, the latter multiply rapidly and may form veritable masses of culture which fill up the proventriculus and even extend forward through the gullet. Fleas showing marked blocking of the proventriculus due to this cause are not prevented from sucking blood, as the pump is situated in the pharynx—they suffer indeed from thirst and bite with abnormal avidity—but the result of the pumping is that the already contaminated œsophagus is simply distended. At the conclusion of the pumping process some of the blood, carrying plague bacilli with it, is forced back into the wound. Success in transmitting plague experimentally to rats by plague-infected fleas was found to be largely, if not wholly, conditioned by a blocked proventriculus. Fleas in this condition are in danger of drying up if the temperature is high and the degree of saturation low, and it is probable that certain features of plague incidence in various parts of India, which appear to be correlated with local meteorological conditions, are in reality primarily dependent on the response of the blocked flea to such climatic variations.

Among other researches which occupied Bacot's attention at this time should be noted his work on the persistence of bacteria in pupæ and imagines of *Musca domestica* raised from larvæ which had been allowed to feed on a test bacillus. In the case of *Musca domestica* certain organisms, such as *B. pyocyaneus* supplied in the larval stage, were able to survive the cataclysm of the metamorphosis and appear in the pupæ and imagines. In the case of fleas, however, Bacot was unable to show that similar organisms could survive the pupal stage, and flea larvæ (*C. fasciatus*), taken from the bodies of mice dead from bubonic plague, and which had had opportunity to feed on fæcal material contaminated with *B. pestis*, showed little evidence of the presence of this organism after dissection and staining of the stomach contents. The conditions in the larval interior of the flea do not appear to be favourable to multiplication of *B. pestis*, just as the larval interior of the fly does not appear to be favourable to the persistence of pathogenic bacterial species, such as *B. typhosus* (Ledingham, Tebbutt, etc.).

In July, 1914, Bacot proceeded to Sierra Leone to take part, as Entomologist, in an investigation into yellow fever, his services being placed at the disposal of the Colonial Office.

The outbreak of war interfered with the carrying out of the scheme of work proposed, but Bacot stayed in West Africa for a year studying the bionomics of *Stegomyia fasciata*, and later published a very complete monograph on the subject.

He returned to this country in October, 1915, and soon found himself immersed in studies on the bionomics of lice with a view to devising efficient methods of sterilising clothing and preventing louse-borne infection at the Front. Bacot's experiments on pediculicides were invariably carried out on himself under conditions similar to those met with in the field. He accepted the position of Honorary Adviser to the War Office on entomological questions, and henceforward he was constantly consulted on matters relating to insecticides.

Opportunity to bring his knowledge of lice and their habits to bear on a problem of great medical and military importance came in December, 1917,

when a Trench Fever Committee formed by the Director-General of the Army Medical Service was constituted under the Chairmanship of Sir David Bruce. Bacot had control of the entomological side of the inquiry, was responsible for the supply of infected lice, and superintended their feeding on trench fever patients and the collection of the louse excreta for further experiment.

In the course of this work Arkwright, Bacot and Duncan confirmed the observation of Töpfer that curious small bodies resembling *Rickettsia prowazeki* in typhus-infected lice were present in the gut lumen of lice fed on trench fever patients. These bodies did not appear in the louse till a period of eight or twelve days had elapsed after a feed on a trench fever patient, and it was only then that such lice were capable of again infecting man.

Trench fever disappeared with the war, and with it the opportunity of continuing research on the Rickettsia bodies associated with it. So many analogies, however, existed between trench fever and typhus fever, alike in connection with louse-transmission generally and association with Rickettsia bodies in particular, that Bacot and his colleagues resorted naturally to typhus research so far as this could be adequately prosecuted in London with passage virus. In 1918, and again in 1920, virus had been obtained from Ireland with which work was undertaken. In the latter year a further opportunity came to Bacot to study this disease when he joined, as Entomologist, the Commission appointed by the League of Red Cross Societies to prosecute typhus research in Poland under the guidance of Drs. Wolbach and Todd. While in Warsaw Bacot was alive to the possibility of finding evidence of the existence of trench or volhynian fever, and such evidence came in a surprising manner. While feeding on his own person lice collected from a public bath-house Bacot developed a sharp fever which necessitated his removal to hospital under suspicion of typhus fever.

During his stay in hospital he continued to nourish these lice and in due course they developed extracellular Rickettsia forms exactly similar to those met with in trench fever lice at home. For some months after recovery he apparently still harboured the trench fever virus in his blood and was able to infect otherwise clean lice by feeding them on himself. The proof that what he had suffered from in Warsaw was trench fever and not typhus was complete enough—a full account of his illness with experimental data appeared in the *British Medical Journal*—and his death from typhus fever almost exactly two years later is further proof, if proof be needed.

The work of the Warsaw expedition has recently been published in book form, and in it the claim of *Rickettsia prowazeki* to represent the actual virus of typhus fever is strongly urged. The chain of evidence, however, was not complete and it was therefore with lively anticipations that Bacot and Arkwright proceeded early in January of this year to Cairo to prosecute further researches into the ætiology of typhus.

Since his visit to Poland, Bacot had been able to practise the intrarectal infection of lice on the lines of Weigl's method, and had succeeded in keeping lice alive for fairly long periods on an intrarectal diet of human blood. By the aid of this new weapon Bacot and Ségal were able to watch the development of Rickettsia in lice thus infected, and to complete a chain of evidence by successfully infecting guinea-pigs with such lice, and thereafter demonstrating the

occurrence of Rickettsia in lice inoculated intrarectally with the blood of these infected guinea-pigs. These most recent experiments by Bacot in conjunction with Ségal and Atkin are in course of publication in this Journal.

No sketch of Bacot's life-work would be complete without some reference to his personal charm, his single-minded devotion to science, and his unfailing desire to be helpful to others. Colleague, acquaintance or casual visitor alike experienced at his hands that old-world courtesy now perhaps rarely seen among us. He leaves indeed a pleasant memory, and his works will live after him.

J. C. G. LEDINGHAM.

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1893–1910.

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THE INFECTION OF LICE (*PEDICULUS HUMANUS*) WITH *RICKETTSIA PROWAZEKI* BY THE INJECTION *PER RECTUM* OF THE BLOOD PLATELETS OF TYPHUS-INFECTED GUINEA-PIGS AND THE RE-INFECTION OF OTHER GUINEA-PIGS FROM THESE LICE.

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FOLLOWING Weigl's (1920) plan of feeding lice by rectal injections (Bacot, 1922), female specimens of *Pediculus humanus* were given an infecting meal of platelet material (Ségal, 1922) obtained by fractional centrifugation of the blood of typhus-infected guinea-pigs (G.P.). These lice were thereafter fed on defibrinated normal human blood by rectal injection, or for a portion of their infective period (in the case of Batches 32 and 33) naturally by allowing them to bite healthy Macacus monkeys. Although the insects fed greedily on the monkey they died within three to four days, apparently because of their inability to digest its blood.

The lice were incubated at 32° C. (90° F.) and fed twice daily for the major portion of the period following their infecting injection, but for some 40 or 56 hours in each week they were keep at room temperature (about 18° C., = 65° F.) and received only one meal during this period.

A number of the lice died showing a heavy bacterial contamination of the alimentary tract; in most *if not all* of the cases the growth of bacteria was almost certainly the actual cause of death.

From the fact that the cases of bacterial contamination were very capriciously distributed among the five batches of infected lice dealt with in this paper, and that the insects contaminated died within four to six days of the infecting meal; there seems no doubt that the source of the contamination was the platelet material* rather than the normal human blood on which they were fed subsequently to the infecting meal. This conclusion is supported by work now in progress.

The development of *Rickettsia prowazeki* was ascertained by dissecting out the guts of specimens which died and making smears of the teased-up guts, or by making smears of recently voided excreta, the former being the more certain of the two methods.

* Accidents which occurred during the preparation of the platelet material in certain cases render it possible that sterility was not absolute.

In a few instances Rickettsia bodies were present in the smears which showed bacterial contamination, but in most cases there had either been insufficient time for them to develop, or else they had been overwhelmed by the more rapidly growing bacteria.

Batch 31.

19 females of *Pediculus humanus* were injected with platelet material obtained from G.P. 72 (Chart 1).

5 specimens	died on the 3rd day.	All negative.
5 ,,	,, ,, 4th ,,	2 of which showed a few thread or short rod forms of *R. prowazeki*.
3 ,,	,, ,, 5th ,,	1 showed doubtful but no definite Rickettsia bodies. 1 showed a few short rod forms (+). 1 showed a few thread forms (++).
2 ,,	,, ,, 6th ,,	1 doubtfully infected. 1 showed thread forms (+).

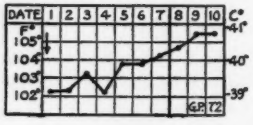

CHART 1.

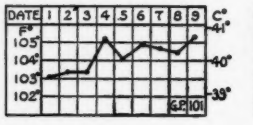

CHART 2.

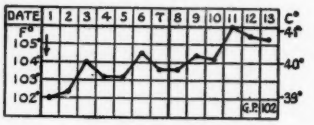

CHART 3.

1 specimen	killed on the 9th day.	Showed short rod and thread forms (++++). An emulsion of this gut was injected into G.P. 101 (see Chart 2).
1 ,,	,, ,, 10th ,,	Showed short rod and thread forms (++++++). An emulsion of this gut was injected into G.P. 102 (see Chart 3). An emulsion of its ovaries was injected into G.P. 103 (see Chart 4).
1 ,,	,, ,, 10th ,,	Fixed for sectioning (unexamined).
1 ,,	,, ,, 10th ,,	Showed short rod and thread forms (++++).

Summary.—No contaminating bacteria. 6 out of 18 specimens examined showed *R. prowazeki*. Guinea-pigs 101, 102 and 103 were injected with emulsions from organs of the infected lice.

Results of injections of louse material into guinea-pigs.—G.P. 101 (Chart 2) injected intra-peritoneally with emulsion of gut. This G.P. was passaged by an injection of 2 c.c. of its blood into G.P. 110 and by an injection of an emulsion of $\frac{1}{3}$ of its brain into G.P. 111. G.P. 110 and 111 showed a positive reaction. G.P. 110 was tested for immunity within sixteen days of its infec-

tion by the injection of an emulsion of $\frac{1}{3}$ of the brain of an infected guinea-pig and gave a positive result. It is possible that this test was performed before immunity had been established, but it has been recorded by Da Rocha-Lima that guinea-pigs that have reacted to an infection of typhus virus have again responded to a second injection.

G.P. 102 (Chart 3) received an injection of an emulsion of the ovaries from the same louse the gut of which was used to infect G.P. 103. This G.P. was passaged by the injection of an emulsion of $\frac{1}{3}$ of its brain into G.P. 122 and 123, both of which showed a positive reaction.

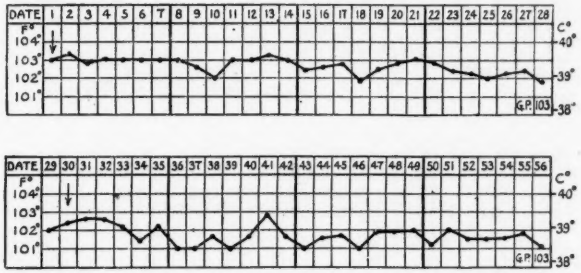

CHART 4.

G.P. 103 (Chart 4) received an injection of the gut of the same louse the ovaries of which were used to infect G.P. 102. G.P. 103 did not react to the injection, and was subsequently tested for immunity on the 30th day by an injection of brain emulsion from an infected G.P. As it failed to react to this we conclude that the animal was naturally immune.

Batch 32.

21 females of *P. humanus* were injected with platelet material from typhus-infected G.P. 84.

3 specimens	died within	3 hours.	Possibly owing to injury during manipulation.
3 ,,	,,	20 ,,	Cause not ascertained; probably injury or bacterial contamination of gut.
2 ,,	,,	3 days.	1 showed a few Rickettsia forms. 1 negative.
2 ,,	,,	4 ,,	The smear preparations were inadvertently destroyed.
5 ,,	,,	5 ,,	3 showed bacterial contamination. 1 showed Rickettsia infection (+). 1 showed Rickettsia infection (++).

Smears made from the excreta of the 6 living specimens on the 7th day showed that some of the insects were heavily infected by *R. prowazeki*. These 6 lice were fed on a healthy Macacus monkey from this date until their death, the two last being killed only when they were too feeble to feed.

1 specimen died on the 9th day. Showed a heavy infection of small forms of *R. prowazeki* (++++).

1 „ (dying) was killed on the 10th day. Showed a heavy infection of threads and small forms of *R. prowazeki* (+++++).

An emulsion of the gut of this louse was injected into G.P. 121 (Chart 5).

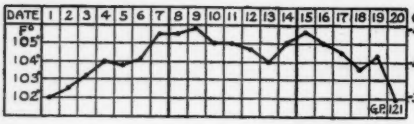

CHART 5.

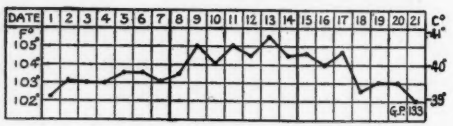

CHART 6.

1 specimen died on the 11th day.
1 „ „ „ 12th „
} Fixed for sections (unexamined).

1 „ killed „ 13th „
1 „ „ „ 13th „
Both heavily infected with *R. prowazeki*, mostly small forms (+++++). Gut emulsions of one injected into G.P. 133 (Chart 6), and of the other into G.P. 134 (Chart 7).

Summary.—9 died from unascertained cause, 3 died showing bacterial contamination, 9 died or were killed showing infection with *R. prowazeki*. Guinea-pigs 121, 133 and 134 were injected with emulsions of guts of these lice.

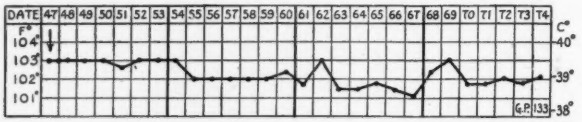

CHART 6A.

Results of injections of louse material into guinea-pigs.—G.P. 121 (Chart 5). Emulsion of gut intra-peritoneally. Died on the 20th day after injection.

G.P. 133 (Chart 6). Emulsion of gut intra-peritoneally. This G.P. was tested for immunity by the injection of ⅓ brains on the 47th day after the first injection (Chart 6A).

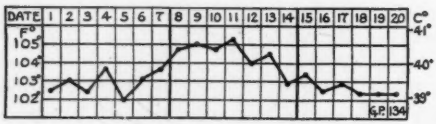

CHART 7.

G.P. 134. Emulsion of gut subcutaneously (Chart 7). This pig was tested for immunity by the injection of ⅓ brain on the 35th day after the first injection, but the animal died on the 14th day without reaction from unascertained cause.

Batch 33.

12 females of *P. humanus* were injected with platelet material from typhus-infected G.P. 87. The preliminary wash in 2 per cent. lysol was omitted in the case of this batch. It is very doubtful, however, if the heavy incidence of bacterial contamination is attributable to this cause.

2 specimens died shortly after injection. From the second day after the infecting meal the remaining 10 were fed naturally by allowing them to suck blood from a healthy *Macacus rhesus* monkey. Within 5 days all had died, showing heavy bacterial contamination of their guts. In every case a few poorly stained Rickettsia forms were observed.

Batch 34.

18 females of *P. humanus* were injected with platelet material from typhus-infected Guinea-pigs 137 and 146.

3 specimens	died within 2 days.	Two were examined; both showed heavy bacterial contamination of the gut.
6 „	„ „ 3 „	All showed heavy bacterial contamination of the gut.
2 „	„ „ 4 „	Both showed heavy bacterial contamination of the gut.
2 „	„ „ 5 „	1 showed heavy bacterial contamination of the gut. 1 showed a rather indefinite infection, probably of *R. prowazeki*.
1 specimen	killed on the 7th day.	Showed slight infection of *R. prowazeki* (+). An emulsion of the gut was infected into G.P. 183 (Chart 8).
1 „	died „ 9th „	Showing heavy bacterial contamination of gut and also poorly stained *R. prowazeki* (++).
1 „	„ „ 10th „	Showing slight infection with *R. prowazeki* (+).
1 „	(dying) was killed on 14th day.	Showing very heavy infection with *R. prowazeki* (++++).
1 „	was killed on 14th day.	Was fixed for sectioning (not yet examined).

Summary.—2 died unexamined; 11 died showing heavy bacterial contamination of gut; 1 died showing heavy bacterial contamination and also infection with *R. prowazeki*; 1 died showing possible infection of *R. prowazeki*; 3 showed definite infection of *R. prowazeki*.

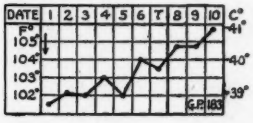

CHART 8.

Result of infecting G.P. 183 *with emulsion of gut.*—G.P. 183 was injected with an emulsion of the gut of a louse of Batch 34 (Chart 8). Material from this G.P. (183) was passaged (injection of an emulsion of ½ of its brain) to Guinea-pigs 201 and 202 (Charts 9 and 10).

G.P. 202 failed to react within 35 days and died before it could be tested for immunity.

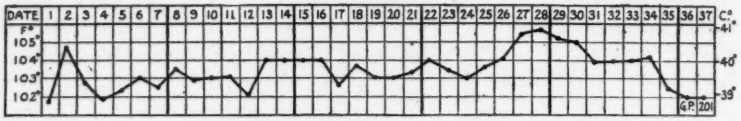

CHART 9.

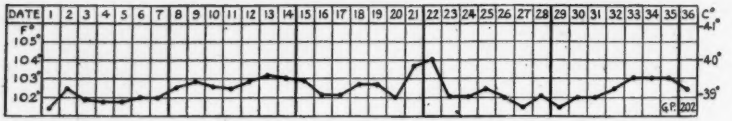

CHART 10.

Batch 35.

17 females of *P. humanus* were injected with platelet material from typhus-infected G.P. 141.

6 specimens died within 24 hours. } All showed a more or less heavy bacterial
1 specimen „ 36 „ } contamination of the gut.

1 „ killed on the 4th day. Showed an indefinite picture; very slight if any development of *R. prowazeki*.
An emulsion of the gut was injected into G.P. 182 (Chart 11).

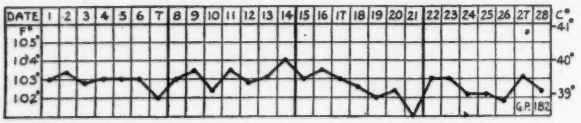

CHART 11.

1 specimen (dying) killed on 7th day. Showed (+ + +) infection of the gut-cells with thread and bacillary forms of *R. prowazeki*.

1 „ killed on the 8th „ Showed threads and small bacillary forms (+ + + +) of *R. prowazeki*.
An emulsion of the gut was injected into G.P. 191 (Chart 12).

1 „ „ „ 11th „ Showed minute bacillary forms of *R. prowazeki* (+ + + +).
An emulsion of gut (+ + + +) was used to infect another batch of lice, and an emulsion of the ovary (+) was used to infect G.P. 198 (Chart 13).

1 specimen (dying) killed on 12th day. Showed minute bacillary forms (+ + + +) of *R. prowazeki*.
An emulsion of the gut (+ + + +) was injected into G.P. 199 (Chart 14).
An emulsion of the ovary (+) into G.P. 198.

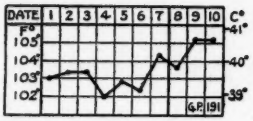

CHART 12.

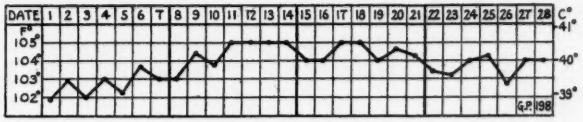

CHART 13.

1 specimen died on the 13th day. Showed minute bacillary forms of *R. prowazeki* (+ + + +).
1 „ killed „ 13th „ Fixed for sectioning (unexamined).
2 specimens died „ 14th „ Showed small bacillary forms of *R. prowazeki* (+ + + +) and few threads.
1 specimen killed „ 14th „ Showed small bacillary and short rod forms of *R. prowazeki* (+ + + +).

Summary.—1 unexamined; 7 died showing bacterial contamination; 1 indefinite picture; 8 showed more or less heavy infection of *R. prowazeki*.

Guinea-pigs 182, 191 and 198 were injected with emulsions of organs of lice of Batch 35.

Results of infecting guinea-pigs and lice with emulsions of organs from Batch 35.—G.P. 182. Infected with emulsion of gut (Chart 11) failed to react.

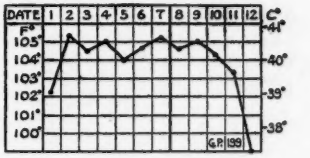

CHART 14.

G.P. 191. Emulsion of gut (Chart 12).

G.P. 198. Emulsion of the ovaries of two lice injected on successive days (Chart 13).

G.P. 199. Emulsion of gut injected into testicle: died on the 12th day (Chart 14).

21 females of *Pediculus humanus* were injected with an emulsion of gut of a louse of Batch 35 and defibrinated human blood (Batch 36).

Smears of lice of this batch showed a more or less heavy infection of *R. prowazeki*. There were no cases of bacterial contamination.

Judging by the previous experience of one of the authors, the time occupied by the development of *R. prowazeki* in the louse when the platelets were used as the infecting material was only about $\frac{1}{2}$ to $\frac{2}{3}$ that required for an equivalent development when the lice were fed on an infected monkey or when whole blood of infected guinea-pigs was injected.

This is, no doubt, due to the greater concentration of the organism in the platelet material than in whole blood, and the time needed for lice to acquire a

heavy infection can be still further reduced by using as infecting material an emulsion of the gut of a heavily infected louse.

Apparently in whole blood the number of infecting units is so few that there are not enough gut-cells infected to enable them to be readily detected even at the end of their necessary incubation period. Later a secondary massive infection of the gut-cells takes place, probably as a result of the rupture of those first infected and the discharge of their contents into the lumen of the gut.

CONCLUSIONS.

(1) The injection of lice (*Pediculus humanus*) with a concentrated emulsion of platelets obtained by fractional centrifugalisations of the blood of a typhus-infected guinea-pig affords a sure and quick method of obtaining the development of *Rickettsia prowazeki* in these insects.

(2) The lice thus infected can be used to convey typhus fever to fresh guinea-pigs.

(3) The parallel development of typhus virus and *Rickettsia prowazeki* in successively passaged guinea-pigs is demonstrated and also in lice infected from guinea-pigs after 23 blood or brain passages.

The following succession has been obtained :

Blood from a human case, Dublin, April 19th, 1921. → Guinea-pigs infected and the virus passaged by blood or brain throughout 18 generations. → *Pediculus humanus* infected by the rectal injection of platelet material obtained from 18th generation showed *Rickettsia prowazeki*. → Guinea-pigs infected with an emulsion of the organs of these lice gave typhus reactions.

Pediculus humanus infected by the rectal injection of platelet material obtained from 18th generation showed *Rickettsia prowazeki*. → Lice infected by the rectal injection of an emulsion of the gut of infected lice through 4 generations of lice showed *R. prowazeki*. → Guinea-pigs infected with an emulsion of guts of lice of the 4th passage from insect to insect showed typhus reactions.

We desire to express our thanks to our colleagues Drs. Ledingham, Arkwright and Atkin for advice and assistance. Our grateful acknowledgments are also due to Dr. Atkin for supplying the virus, which he had already passaged through 8 generations of guinea-pigs before we started with the strain.

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VARIATIONS IN THE DIASTATIC POWER OF THE URINE IN RELATION TO ITS REACTION, WITH A SUGGESTED METHOD FOR THE ESTIMATION OF THE DIASTASE CONTENT.

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Received for publication March 14th, 1922.

SINCE the discovery by Wohlgemuth (1908) of a diastatic enzyme in the urine, its estimation has taken a very important place among the chemical aids to diagnosis, and has proved itself to be of great value in the detection of certain diseases, more especially in pancreatic and renal lesions.

In certain cases of nephritis (Wohlgemuth, 1909) the diastatic power of the urine is subnormal, whereas in lesions of the pancreas (Wohlgemuth, 1910) it is very much higher than normal. It has been said to be high in eclampsia (Wallis, 1920), and the writer has demonstrated that acute rickets is accompanied by a very high diastatic power. From what has been said it becomes obvious that the reaction is of considerable importance, both from the clinical and from the research aspects. Probably the diseases mentioned above vary the urinary diastase content by acting in one of two ways: (*a*) by altering the production of diastase (pancreatitis), or (*b*) by interfering with the excretion into the urine (nephritis). Now if the reaction is to be of value in the diagnosis of these diseases, then the above two conditions, and these alone, must be allowed to operate. It is the object of this paper to show that other purely chemical factors can influence the reaction, and if these are not eliminated, the value of the test is materially diminished.

By far the most important of these factors is the hydrogen ion concentration, and, to a lesser extent, the salt concentration of the urine (Evans, 1912). In the original technique (Corbett, 1913; Stocks, 1916) no account is taken of either, though they may vary to such a degree as seriously to interfere with the estimation of the diastase.

THE REACTION OF THE URINE IN RELATION TO DIASTATIC POWER.

Many workers have called attention to the importance of the effect of hydrogen ion concentration on enzyme action, and research on this problem has proved that for each enzyme there is a definite optimum pH, and that this optimum reaction differs for different enzymes.

Michaelis and Peckstein (1914) showed that salivary diastase formed

compounds with salts, such as chlorides, sulphates, nitrates and phosphates, that each of the resulting compounds was amyloclastic, and that each had an optimum pH. The phosphate, sulphate and chloride compounds worked best at pH 6·1, 6·1 and 6·7 respectively, and it was found that the chloride compound could be replaced by excess of phosphate, and similarly the other compounds were interchangeable. The metallic radicles had little or no effect. In common with all enzymes any alteration of hydrogen ion concentration, either above or below the optimum, led to a decrease in its amyloclastic action.

These researches suggested an inquiry into the effect of reaction upon urinary diastase, and the first investigation carried out was the fixing of the optimum pH. Since buffer solutions of varying reaction are so easily made with mixtures of phosphates, it was decided to determine the optimum pH of the phosphate diastase compound in urine. This was done by taking a quantity of urine and diluting it 1 in 5 with buffer solutions of varying pH. The diastatic power of these mixtures was then determined in the way described below for the new technique. A number of urines were treated in this manner, and the results, calculated as described on p. 135, are seen in Table I. As in the case of salivary diastase, 6·1 appears to be the optimum pH for the phosphate diastase compound of the urine.

TABLE I.—*Optimum pH of Diastatic Power of Urine.*

Urine no.	Diastatic power at pH.						
	4.	4·5	5.	5·5.	6·1.	6·5.	7·0.	7·5.
1	10	25	25	28	33	28	20	10
2	0	0	10	20	20	10	6·6	0
3	0	0	6·6	10	28	25	20	10
4	0	6·6	10	20	28	25	20	0
5	6·6	10	20	25	28	25	20	10
6	10	20	20	25	33	25	20	15
7	0	6·6	10	20	20	10	6·6	0
8	25	33	66	70	80	50	25	10
9	0	10	20	28	33	28	20	10
10	0	6·6	10	20	28	20	20	10
11	10	20	20	28	33	28	28	20
12	0	0	10	20	28	20	20	10
13	10	10	20	20	28	20	20	10
14	20	28	28	33	50	33	33	20
15	0	10	20	20	33	20	10	0
16	10	10	15	20	25	20	20	15

Now, suppose that three urines, all containing the same quantity of diastase, but with reactions of pH 4·5, 6·1 and 7·0, are examined for diastase content by means of the old technique, all three will give different values; the middle one, being at the optimum pH, will give a much higher value than the other two, and thus it is obvious that all urines must be brought to the optimum reaction before being tested for diastatic power.

After a great many trials of different methods the following was found to be by far the easiest and best.

NEW METHOD PROPOSED.

Solutions required.—(1) A 0·2 per cent. starch solution. This is best made up by adding the weighed quantity of starch pinch by pinch to the requisite volume of distilled water, and stirring until an even suspension is obtained. The mixture is then slowly brought to the boil, stirring all the time. After boiling for a short time the solution becomes opalescent, when it is cooled and the volume is made up to the correct amount. By this means no troublesome gelatinous masses form and the solution is quite uniform. Preferably this solution should be made up fresh each day and should never be kept more than a few days.

(2) Phosphate buffer solution. This is obtained by mixing 15 c.c. of Sörensen's solution A with 85 c.c. of solution B.

Solution A is made by dissolving 11·876 grm. of Na_2HPO_4, $2H_2O$ in one litre of boiled distilled water, the solution being kept in a paraffin-coated bottle.

Solution B is made by dissolving 9·078 grm. of KH_2PO_4 in 1 litre of boiled distilled water, and is stored in a paraffin-coated bottle.

The resulting solution should have a pH of 6·1.

In the whole of this investigation the buffer solutions have been checked by the hydrogen electrode.

Method.—1·5 c.c. of urine are added to 6 c.c. of the buffer solution. The resulting solution is well shaken in order to ensure uniform distribution. A series of test-tubes are then put up, each being filled as follows:

	Test-tube No.						
	1.	2.	3.	4.	5.	. . .	12.
0·2 per cent. starch solution . .	1 c.c.	1 c.c.	1 c.c.	1 c.c.	1 c.c.	. . .	1 c.c.
Buffered urine .	1·5 c.c.	1 c.c.	0·5 c.c.	0·45 c.c.	0·4 c.c.	. . .	0·05 c.c.
Distilled water .	0·5 c.c.	1 c.c.	1·5 c.c.	1·55 c.c.	1·6 c.c.	. . .	1·95 c.c.

Short tubes are used ($\frac{1}{2} \times 4''$), as with these there is less liability for drops of the various ingredients to stick to the sides of the tube, and, by dropping down when the iodine is added, spoil the end-point. The urine and the fractions of a c.c. of water are added first, followed by the starch solution. It now remains to add rapidly 1 c.c. of water to each tube except No. 1. The sides of the tube are deliberately washed down into the bottom with this distilled water. By adding the solutions in the above order all the tubes get about the same digestion time. The tubes are incubated for half an hour at 37° C., cooled, and N/50 iodine added. As small a quantity as will just give a faint colour is used, excess making the end-point much more obscure.

Calculation of results.—Suppose the tube just not showing a mauve tint, *i. e.* the one where the starch was just digested, contained 0·5 c.c. of the diluted urine. Therefore 0·5 c.c. of diluted urine, or 0·1 of the undiluted urine, just digests 1 c.c. of 0·2 per cent. starch, or 2 c.c. of 0·1 per cent. starch. Since the number of Wohlgemuth's units is given by the number of

c.c. of 0·1 per cent. starch solution digested by 1 c.c. of urine, it is obvious that in the above case 20 units of diastase were present. 0·2 per cent. starch solution is used instead of 0·1 per cent. in order that the total volume may be kept to 3 c.c. as in the original technique. If greater volumes are used the end-point becomes indistinct.

Distilled water has been substituted for saline usually used as the fluid for making up to the constant volume. It is difficult to see the reason for using saline, and the writer has found that it tends to obscure the end-point.

In Table II will be seen the comparison of the old and modified technique on 50 urines. It will be seen that the statements already made are fully borne out, and that unless the reaction of the urine is pH 6·1 the diastase is hampered, and an incorrect estimate is obtained by the old technique.

TABLE II.—*Diastatic Power of Urine according to Old and New Method.*

Urine no.	Ordinary method.	New method.	pH of urine.	Urine no.	Ordinary method.	New method.	pH of urine.
1	28	33	6·5	26	25	50	4·5
2	5	10	4·5	27	2	6·6	4·5
3	20	30	7	28	6·6	10	5·5
4	5	10	4·5	29	2	10	4·5
5	5	10	5	30	10	25	4·5
6	2	6·6	4·5	31	2	10	5
7	28	30	7	32	2	10	5
8	28	30	7	33	20	66	4·5
9	6·6	10	6·8	34	2	6·6	5
10	10	22	6·8	35	20	40	4·5
11	10	6·6	6·8	36	2	10	5
12	10	20	7	37	22	30	5
13	5	6·6	4·2	38	22	25	5·5
14	2	10	4·2	39	6·6	10	6·8
15	150	150	6	40	20	33	4·5
16	10	10	6	41	6·6	10	5·5
17	10	10	6	42	25	30	5
18	150	150	6	43	25	30	5·5
19	10	10	6	44	5	20	8
20	20	25	5·5	45	2	6·6	7
21	10	10	6	46	2	6·6	7
22	10	10	6	47	2	6·6	8
23	25	28	6·8	48	2	10	7·5
24	10	10	6	49	2	10	8
25	10	20	4·8	50	0	25	8·5

It has been stated that ammoniacal decomposition has no effect on the diastase reaction. The last seven urines in Table II were ammoniacal, and it will be seen that, owing to their high alkalinity (pH 7 to pH 8·5), they gave abnormally low readings by the old method, but quite normal figures by the new. This obviously might lead to errors in diagnosis. Also, if urine which contains the slightest deposit be allowed to stand over-night the diastase sinks to the bottom, being absorbed to the surface of the deposit. A series of urines

were left to sediment over-night, and a diastase reaction was carried out on the supernatant part of the liquid, and on the sediment.

From Table III it will be seen that there is very much more diastase in the bottom of the jars than the top. That this was only due to an adsorption on to the deposit was shown by centrifuging clear urine for a long time, when no sedimentation of the diastase could be detected.

TABLE III.—*Absorption of Diastase by Urinary Deposits.*

Urine no.	Before centrifugalisation.	After centrifugalisation.	
		Supernatant fluid.	Fluid from bottom of tube.
1. Marked deposit .	33	2	25
2. ,, ,, .	10	25	28
3. ,, ,, .	20	0	10
4. ,, ,, .	28	10	25
5. ,, ,, .	50	0	60
6. No deposit .	20	20	20
7. ,, ,, .	10	10	10
8. ,, ,, .	6·6	6·6	6·6

SUMMARY.

(1) The original technique for the diastase reaction is criticised because it takes no account of the varying reaction of normal urine.

(2) It is proved that the optimum reaction for urinary diastase in the presence of phosphate was pH 6·1, as in the case of the salivary diastase compound.

(3) A modified technique is described whereby the urine is diluted with a phosphate buffer solution, thus bringing all urines to the optimum pH before its starch-digesting power is tested.

(4) Ammoniacal decomposition, by making the urine more alkaline, decreases the diastatic power as determined by the old method, but has no effect on the method suggested.

(5) Diastase tends to cling to urinary deposits, hence all urines should be well shaken before the estimation is performed.

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AN INVESTIGATION INTO THE ÆTIOLOGY OF DENTAL CARIES. I: THE NATURE OF THE DESTRUCTIVE AGENT AND THE PRODUCTION OF ARTIFICIAL CARIES.

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CONSIDERING the wide-spread and almost universal distribution of dental caries, it is not a little surprising that we have no definite knowledge of the causal agent or agents. Numerous theories have been propounded in explanation, details of which are to be found in the various monographs which have appeared from time to time. It is therefore not proposed to deal with these views individually here. Perhaps the parasitic theory, or, as it is more aptly expressed by Miller (1890), the chemo-parasitic theory, has received most support.

The chemo-parasitic theory of dental caries may be regarded as having originated with Miller's brilliant researches. The purely inflammatory view is of course much older. Miller regarded acids produced by the fermentative action of bacteria as the primary factor in dental decay. He, however, lived in advance of his time; his bacteriological technique was exact, but bacteriological knowledge was not sufficiently advanced to profit by his researches or to identify the organisms he found in the lesions. Many of the bacteria observed by him failed to grow on the media at his disposal, so that much of his study was purely morphological. He definitely showed that mouth bacteria, by their action on carbohydrates, could produce sufficient acid to injure teeth. Since Miller's time most of the work seems to have consisted in enumerating the various bacteria found in the mouth and in dental caries—researches which might be more aptly described as investigations into the bacterial associations of dental caries. Miller enumerated a very large number of bacteria and classified them according as to whether he was able to grow them or not. Goadby (1903) in his valuable monograph described some eighteen types, which he subdivided into acid-producing types and dentine liquefiers. Kligler (1915) identified ten different groups. Howe and Hatch (1917), although no exact details or actual figures are given, consider that two types of bacteria, which they group together under the name "Moro-Tissier," are most commonly associated with dental caries.

NATURE OF THE DESTRUCTIVE AGENT.

Decalcification.

In taking up this research on dental caries we were of opinion that if bacteria played an important *rôle*, then it was necessary to find micro-organisms which were capable of softening enamel, which process histo-pathological research has shown to be the initial lesion in caries. For the present, of course, we do not intend to deal with any of the predisposing causes, although the part played by structural deformities, whatever be their origin, cannot be denied. We have already noted that Miller considered that the production of acid by bacteria was the initial process. We therefore have endeavoured to discover a bacterium or group of bacteria present in dental caries which can produce sufficient acid to decalcify enamel and dentine. Once decalcification has been produced, the path is left open for almost any micro-organism to invade the dentine.

It is reasonable to suppose that the bacteria which produce large quantities of acid will be able to live in relatively high concentrations of acid. Therefore, bacteria which are able to decalcify enamel must be able to live in a very acid medium. This is briefly the thesis on which we have based this research and the basis of our technique to discover the bacterium.

The first procedure was to determine what degree of acidity is required to decalcify tooth enamel. This can be arrived at in two ways—experimentally and theoretically.

Experimentally the simplest procedure is to place normal teeth (*i. e.* teeth showing no caries) into solutions of different degrees of acidity. In our experiment we used acid nutrient broths of different pH values. The acid broths varied from pH 5 to pH 1, and the teeth were left in for thirty-four weeks. The teeth had previously been sterilised in the broth by autoclaving. At the end of the period the degree of whitening or opacity of the enamel was noted. It was found that no change was to be seen in those teeth placed in broths of pH values higher than 4, and only such a minute trace as to be negligible in pH4 (Fig. 1).

The theoretical determination is much more difficult, as the formulæ have all been constructed to deal with elements in solution. Dealing with the carbonate radicle, it is possible to arrive at an approximate value by the calculation of the pH value of a saturated solution of carbonic anhydride. A saturated solution such as might occur in the mouth would give a solution corresponding to about N/1000.

From the formula $\sqrt{K_1 \times 1}$, where K_1 = the dissociation constant, we get a pH value of 4·8 for such a solution.

The chemical affinity of acids in equivalent solutions is proportional to the dissociation constants, therefore we can assume that the acidic values must be in excess of this pH value before the carbonic oxide can be displaced from a compound, as it is reasonable to suppose that teeth are not affected by the CO_2 of the saliva.

Technique of Isolation.

Carious teeth were obtained from the Dental Out-Patient Department and from two private practices in order to include as many different types of

mouth conditions as possible. A tooth was held in sterilised forceps and passed through the flame a few times; with a sterile scalpel the superficial part of carious dentine was removed and the deeper part emulsified in broth pH 7·6. After twenty-four hours' incubation broths of varying degrees of acidity were heavily inoculated, and from these agar plates were inoculated after twenty-four or forty-eight hours' incubation. At first the acid broths used varied from pH 7 to pH 4·5, but as a large variety of organisms grew in each tube higher concentrations of acid up to pH 1 were tried. Bacilli were isolated with some frequency from pH 3 broths, and on one occasion from pH 1, but on the whole we found there was no need to employ such degrees of acidity. Later the carious dentine was emulsified in pH 6 broth direct, and after twenty-four hours' incubation the more acid broths of pH 4·5 to pH 3 were inoculated. The most successful method, however, was to emulsify the carious material in pH 3·5 broth in the first case, as in this the vast majority of tooth organisms fail to grow.

Nature of Micro-organisms Isolated.

The organisms isolated by the above method fall, morphologically, into two main groups: (*a*) Type I, a long thin bacillus, 0·75 × 2–3 μ, which occurs singly, in pairs or chains, having a marked tendency to parallelism or palisade formation in dried films (Figs. 2 and 3); and (*b*) Type II, a shorter bacillus, 0·75 × 1–2 μ, usually occurring in chains (Fig. 4). After prolonged subculture on agar Type I fails to form chains. Both organisms are non-motile and Gram-positive; both are aërobes and facultative anaërobes (in gelatin stab cultures there is equal growth at the top and the bottom). In broth there is in most cases uniform turbidity, but in a few cases the growth settles to the bottom. On ordinary agar the colonies may appear as minute points after twenty-four hours' incubation, but often do not become visible till after forty-eight hours; they are small, round, greyish and opaque, with a finely granular appearance under the low power and a regular outline (Fig. 5). In size the colonies are about 0·5–1·0 mm. in diameter; on serum agar rather larger, up to 2·0 mm. In a gelatin-agar shake culture the colonies have a rough biconvex appearance which might be described as being "tam-o'-shanter" shaped (Fig. 6).

When first isolated subculturing should be carried out once a fortnight, as the organisms have a tendency to die out, but after prolonged subculture once a month is sufficient.

Type I was isolated in pure culture from the deeper layers of carious dentine from 38 out of 50 teeth examined, and Type II from 18 teeth out of 50. In some cases the growth of mouth organisms in the original broth culture outgrew the above two types to such an extent that they were not isolated although morphologically similar organisms were seen in films; inoculation into acid broth direct obviates this difficulty. Including these cases, Type I "occurred" in 44 cases, *i.e.* 88 per cent., and Type II in 21 cases, *i.e.* 42 per cent. In some cases only one type was isolated from each tooth, in others both types; one or other or both types were isolated in pure culture from 88 per cent. of cases and "occurred" in 96 per cent.

As regards the fermentation of sugars, of the 38 strains of Type I isolated 33 formed acid and no gas in glucose and lactose, no acid nor gas in saccharose

and acid and clot in milk. The milk usually became acid on the second day and was firmly clotted on the third, with all except the top quarter to half an inch completely decolourised. Four of the remaining five strains formed acid in saccharose and glucose, but differed in their reactions in lactose and milk; one strain formed acid in glucose alone. The action of Type II on sugars is nothing like so constant; seven out of the eighteen strains isolated formed acid and no gas in glucose, lactose and saccharose, and acid and clot in milk, the effect on the last-named being similar to that of Type I. Three strains formed no clot in milk after prolonged incubation, and two strains had no effect at all on milk. The remaining six strains varied considerably in their reactions (Table I).

TABLE I.—*Sugar Reactions.*

	Glucose.	Lactose.	Saccharose.	Milk.	Gelatin.	Indol.	No. of strains.
Type I.	Ac.	Ac.	0	Ac. Cl.	0	0	33
	Ac.	Ac.	Ac.	0	0	0	2
	Ac.	0	Ac.	Ac.	0	0	1
	Ac.	0	Ac.	0	0	0	1
	Ac.	0	0	0	0	0	1
Type II.	Ac.	Ac.	Ac.	Ac. Cl.	0	0	7
	Ac.	Ac.	Ac.	Ac.	0	0	3
	Ac.	Ac.	Ac.	0	0	0	2
	Ac.	Ac.	0	Ac.	0	0	1
	Ac.	0	Ac.	0	0	0	2
	Ac.	0	0	Ac. Cl.	0	0	1
	Ac.	0	0	Ac.	0	0	1
	Ac.	0	0	0	0	0	1

In no case was gelatin liquefied by either type, nor was indol formed; twenty-six strains were also inoculated into dextrin and dulcite without the formation of acid or gas.

We propose to give the name *B. acidophilus odontolyticus I* and *II* to these bacilli.

Occasionally other organisms were isolated, but with no regularity.

Relation of the Two Types to other Bacteria found in Dental Caries.

Morphologically Type I very closely resembles one of the organisms illustrated by Miller in an excellent micro-photograph of artificial caries. Howe and Hatch state that the predominating group of organisms in dental caries is the Moro-Tissier group. *B. acidophilus* (Moro) as depicted by them bears morphological resemblance to Type I. The sugar reactions, however, do not agree, since he states that most strains ferment saccharose; the action on milk is not mentioned, but Moro (1905) states that cow's milk is coagulated while human milk is not.

Kligler, working on oral micro-organisms, isolated fifty-eight strains of *B. acidophilus* (Moro), which he states ferments glucose readily with the formation of a considerable quantity of acid, lactose not so readily and

saccharose not constantly. As regards milk, the lower part is coagulated first and litmus milk becomes decolourised. Summarised, the following are the reactions of the strains isolated by him:

No. of strains.	Glucose.	Lactose.	Saccharose.	Milk.	Gelatin.	Indol.
3	+	0	0	0	0	0
35	+	+	0	A.C.	0	0
20	+	+	+	A.C.	0	0

That is, 60 per cent. agree in the sugar reactions with our Type I.

The Occurrences of Types I and II in "Normal" Mouths.

In order to form some idea as to the frequency of occurrence of decalcifying organisms in the mouths of persons who had at one time or another suffered from caries, ten persons thoroughly rinsed their mouths with 10 c.c. of normal saline, and the fluid was collected and deposited into an equal quantity of double-strength broth. From these cultures Type I organisms were isolated in 30 per cent. of cases and Type II in 20 per cent. This experiment will be further described in a later paper, along with the exact relation of the bacilli and their distribution.

Agglutination.

To test agglutination properties, two strains of Type I were inoculated repeatedly into rabbits; each strain agglutinated with its own antiserum up to a dilution of more than 1 in 2560. Sixteen strains were put up against serum A, and 14 against serum B, with the following results:

		No. of strains agglutinating with various serum dilutions.						
	No. put up.	1/40	1/80	1/160	1/320	1/640	1/1280	1/2560
Serum A	16	16	15	12	12	10	8	9
Serum B	14	14	14	13	8	5	5	6

These results include strains of both types, but in each case the organisms which were agglutinated in the higher dilutions belonged to Type I.

Final pH Values of Cultures.

It has been stated above that we found that a concentration of acid greater than pH 4 was necessary to effect decalcification of enamel, therefore it was essential to discover whether the organisms isolated were capable of forming this amount of acid by the fermentation of carbohydrates. Nine strains picked at random were inoculated into glucose broth of pH 7·6 and placed in the incubator until they died out, and the final pH values were estimated on the hydrogen electrode; these varied between pH 3·4 and pH 2·2, the average being pH 2·75. It thus appears that organisms of either Type I or Type II are able to elaborate enough acid from the fermentation of carbohydrates to attack the enamel of teeth. A seven days culture of *B. coli* in glucose broth gave a final pH value of 4·0.

PRODUCTION OF ARTIFICIAL CARIES.

Decalcification of Enamel.

Attempts were next made to produce caries artificially by the action of the organisms isolated. Non-carious teeth were placed in broth and 2 per cent. glucose broth in 3″ × 1″ tubes and sterilised; various strains were then inoculated into the broth, *S. salivarius* in both ordinary broth and glucose broth and *B. coli* in glucose broth being used as controls. Every eighth day the teeth were removed and placed into fresh uninoculated broth under sterile precautions, so as to approximate somewhat to the conditions in the mouth and prevent the teeth remaining continually in an acid medium. As pointed out by Miller, the first sign of caries is the loss of transparency of the enamel, which becomes opaque; this he attributes to pure decalcification. This opacity is clearly seen in the tooth in Fig. 7, which had been in a broth culture of Type I for seven weeks. Figs. 8 and 9 show low- and high-power magnifications of a longitudinal section from a tooth, the upper half of which had been in a glucose broth culture of Type I for seventeen and a half weeks. The organisms have penetrated the dentinal tubules for a considerable distance *from the pulp cavity side*, which fact proves that the appearance is artificial, since "natural" caries never starts from the pulp cavity and works outwards. In the plates not only can the organisms be seen passing down the tubules, but also a large liquefaction focus can be seen. Fig. 10 is another tooth in which the dentinal tubules have been cut transversely; some of these are seen to be packed with organisms and dilated to a far greater diameter than the empty tubules, which have simply undergone decalcification during the process of embedding. This tooth shows the result of eleven weeks in a glucose broth culture. In all cases observed up to the present the first sign of caries—that is, the opacity of the enamel—occurs on the cutting edge of incisors or canines and on the tip of the cusps of molars and premolars; this fact was also noted by S. P. Mummery (1909–10) when working with teeth suspended in 0·075 per cent. lactic acid. The control teeth in cultures of *S. salivarius* show no macroscopical changes after five months, nor does the control in a culture of *B. coli* after two and a half months; these experiments are being allowed to continue yet further. All the teeth in broth cultures show some slight opacity of the enamel. Further experiments are also being carried out in glucose broth with the lower part of the tooth sealed over in order to produce caries through the enamel alone. Commencing caries of the dentine from the enamel side is indicated in Figs. 12 and 17. Fig. 11 represents a section of a tooth with the tubules cut transversely, showing the effect of placing decalcified non-carious dentine in a broth culture of *S. aureus* for three weeks. No organisms can be seen but the proteolytic enzyme has had a very marked effect, producing enormous cavities in the dentine, far larger than the liquefaction foci which appear in sections of "natural" caries. For the sections and micro-photographs in Figs. 12 to 19 we are indebted to Mr. J. Howard Mummery; these teeth had been in glucose broth cultures for fourteen weeks.

Penetration and Liquefaction of Decalcified Dentine.

Although in no case was gelatin liquefied nor indol formed by any of the organisms isolated, Figs. 8, 9 and 16 clearly show the liquefaction foci as described by Miller. After completely blocking the tubules the organisms have produced large cavities in the decalcified dentine in several places. It thus appears that when completely deprived of carbohydrates the organisms isolated can exert a liquefying action on the collagen matrix of the dentine, although as yet we have been unable to obtain any action on gelatin.

Animal experiments are being carried out, the results of which will be discussed in a later paper.

DISCUSSION OF RESULTS.

In order to facilitate the correlation of our results with the findings in natural caries a short account of the natural process may not be out of place. The main histological features of caries are definite in type and well known.

Dental decay is, with a few exceptions, universally present in both primary and secondary dentition. The condition commences in the crowns of the teeth usually at the points of contact or in the fissures. Caries of the enamel appears as a white opaque area; this is soon followed by fracture of the enamel prisms, disintegration of the enamel and exposure of the dentine. The dentine is then involved, decalcified, softened and destroyed. Microscopically the bacteria can be seen to have penetrated the dentinal tubules, which are enlarged and breaking down with intercommunicating areas. Ground sections of carious teeth at different stages of the disease show the whole process very clearly. There are, however, variations according to the structure of the teeth.

In many of these preparations bacilli morphologically resembling those we have described are seen alone or with other bacteria. Some of Miller's original photographs show this very clearly.

Enamel changes in artificial caries.—The primary lesion produced by prolonged contact with cultures of our micro-organisms is a whitish opacity similar to that seen in natural caries. Sections show that this change is not uniform in that in certain areas the penetration is much more marked than in others; this is clearly seen in Figs. 12 and 17—sections of a tooth which had been in a broth culture for seven weeks. The sections also show slight undermining of the enamel. Under the microscope the enamel prisms are granular and in places fragmented.

Dentine changes in artificial caries.—Miller noted that the first change was an area of decalcification in advance of the organisms. Fig. 17, a ground section, shows staining of the decalcified dentine, whilst the infected dentine is still more deeply stained. So far we have not been able to get a good specimen showing this passage from the enamel side, but this is mainly due to the fact that we did not leave the teeth long enough in contact with the cultures.

The characteristic mode of spread, however, is well shown in Figs. 8 and 9, and only differs from true caries in that the process started from the pulp surface. Microscopically the bacilli are found to be spreading down the dentinal tubules in a manner identical with the natural process. There is

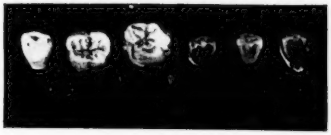

FIG. 1.
Teeth placed in acid broths of pH 5·0 to pH 1·0 to show the acidity at which enamel is attacked. Those in pH 1, 2, 3, enamel attacked; those in pH 4, 4·5, 5, enamel not attacked.

FIG. 2.
Type I bacillus; smear preparation from agar slope culture, showing palisade and chain formation. Gram. × 1000.

FIG. 3.
Same description as Fig. 2.

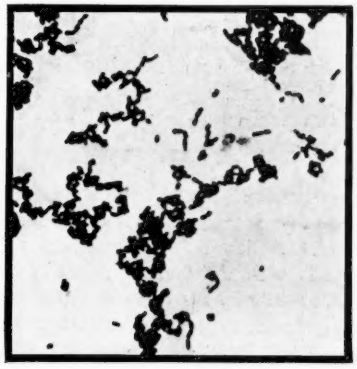

FIG. 4.
Type II bacillus; smear preparation from agar slope culture. Gram. × 1000.

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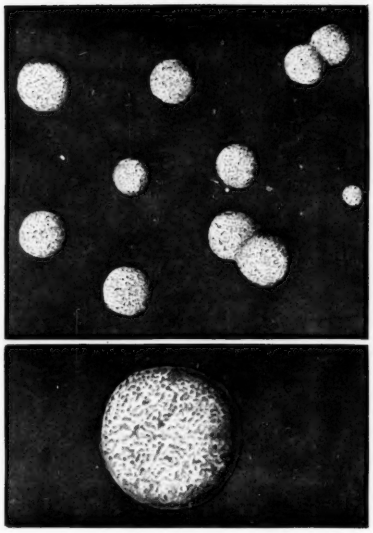

FIG. 5.
Surface colonies of Type I on agar, showing finely granular appearance. × 18 and × 45.

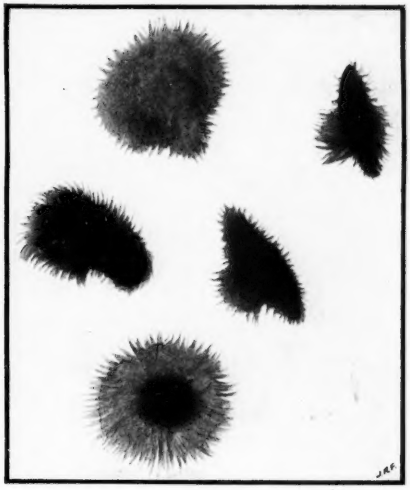

FIG. 6.
Colonies of Type I in gelatin-agar shake culture; "tam-o'-shanter" colonies. × 45.

FIG. 7.
Artificial caries, showing opacity of enamel. Tooth in broth culture of Type I bacillus for 7 weeks.

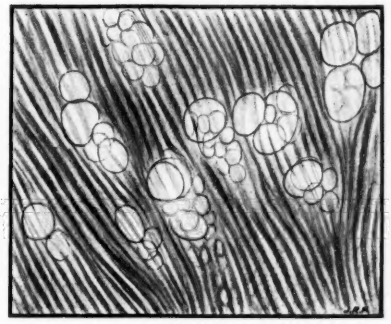

FIG. 11.
Transverse section of dentinal tubules to show the effect of liquefaction of decalcified dentine by *Staphylococcus aureus*. (Zeiss obj. $\frac{1}{8}$ in., oc 4.)

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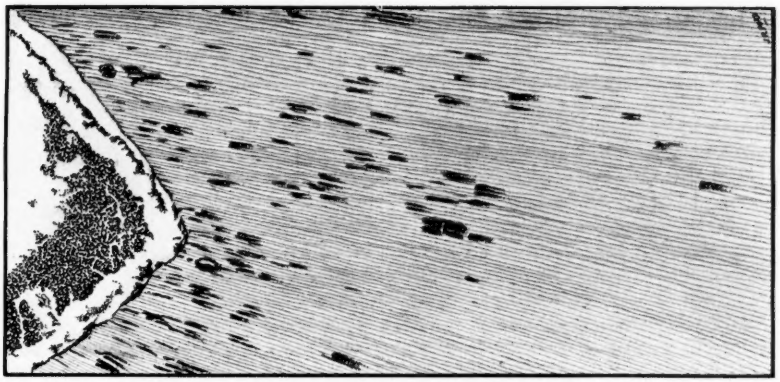

FIG. 8.
Artificial caries; organisms in the dentinal tubules and liquefaction focus. Longitudinal section. (Zeiss obj. $\frac{1}{8}$ in., oc. 4.)

FIG. 9.
Same specimen as Fig. 8. (Zeiss obj. $\frac{1}{12}$ in., oc. 4.)

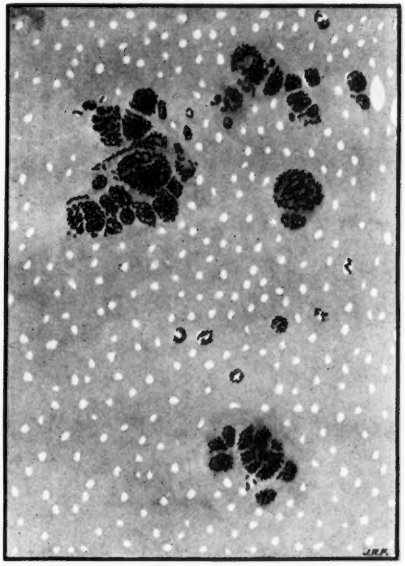

FIG. 10.
Artificial caries; tubules blocked with organisms and dilated. Transverse section. (Zeiss obj. $\frac{1}{12}$ in., oc. 4.)

McIntosh, James and Barlow. Dental Caries.

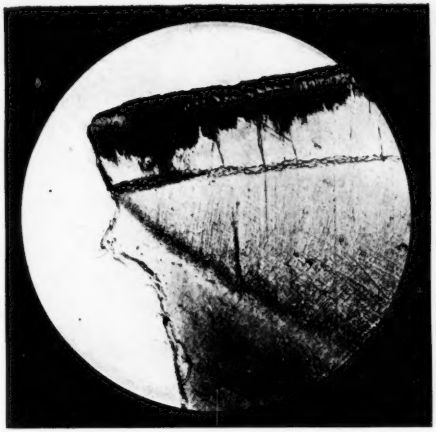

FIG. 12.
Artificial caries; ground section, showing caries of enamel and organisms passing along the amelo-dentinal junction. × 30.

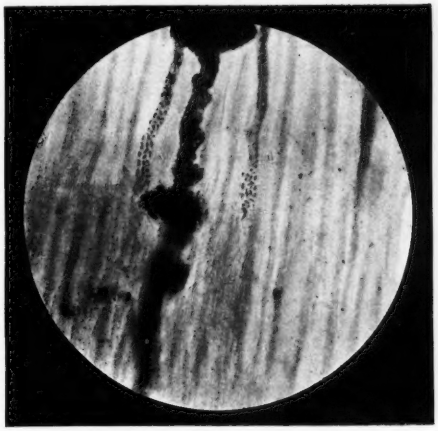

FIG. 13.
Section of artificial caries; individual bacilli in the dentinal tubules, also liquefaction focus. × 700.

FIG. 15.
Artificial caries; section showing spread of bacilli along the dentinal tubules, which are considerably dilated. × 40.

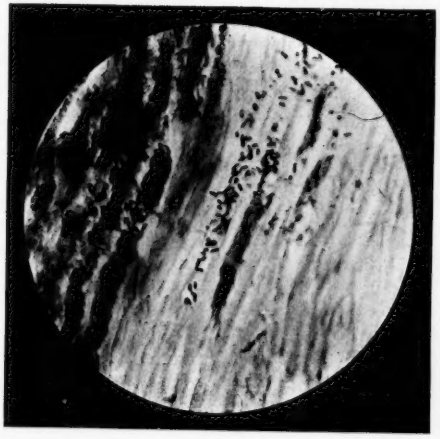

FIG. 14.
Same description as Fig. 13. × 1000.

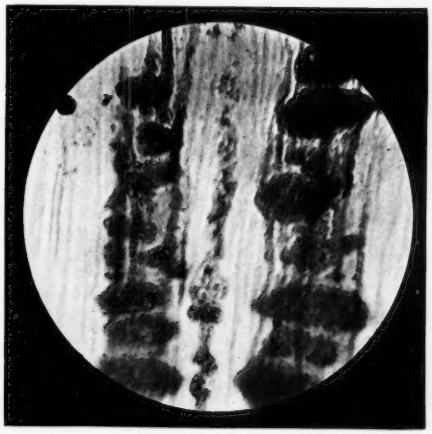

FIG. 16.
Section of artificial caries; large liquefaction foci. × 400.

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FIG. 19.

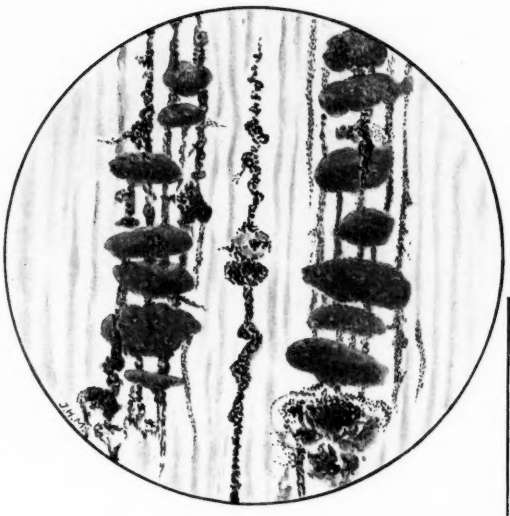

FIG. 19.
Artificial caries; organisms in the tubules and large liquefaction foci. Polychrome methylene blue. × 400.

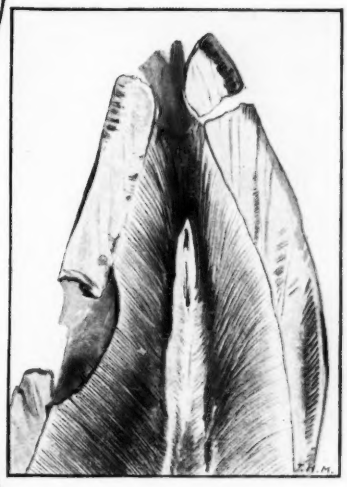

FIG. 17.
Ground section showing caries of the enamel and the undermining of the enamel by the organisms, and commencing destruction of the dentine. × 10.

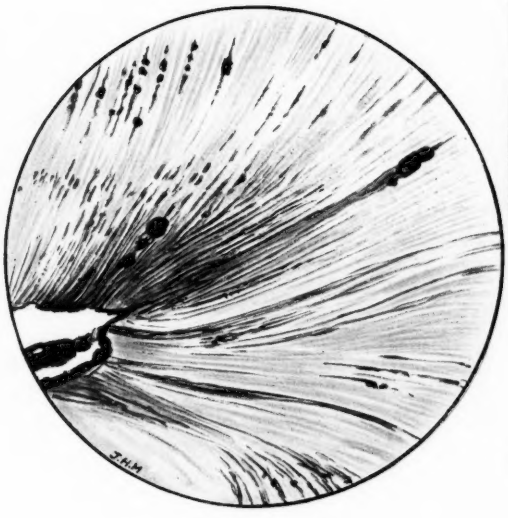

FIG. 18.
Artificial caries starting from the pulp cavity; tubules packed with organisms and showing liquefaction foci. Polychrome methylene blue. × 40.

FIG. 18.

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also a true widening of the tubules with destruction and formation of liquefaction foci. Those foci stain differently with polychrome methylene-blue from the tubules merely blocked with organisms, the former being greenish in colour and the latter blue (Fig. 19).

The production of liquefaction foci strongly supports the ætiological importance of the bacilli we have described. In addition to producing sufficient acid to decalcify teeth and to initiate dental caries they can liquefy dentine. In natural caries, however, it is probable that this liquefaction process may be aided by a secondary infection of the dentine by proteolytic bacteria, although actively proteolytic bacteria such as the anaërobe *B. sporogenes* were never found. Placing decalcified dentine in cultures of proteolytic bacteria produced an extreme degree of proteolysis with the tubules all widely dilated (Fig. 11), and if the action be prolonged the dentine is completely dissolved. Such a condition is never seen in natural caries.

In a further paper we propose to deal with some of the secondary factors in the production of dental caries.

CONCLUSIONS.

(1) The examination of selected carious material showed the constant presence of a definite type of bacillus.

(2) The bacilli are capable of producing a high degree of acidity by the fermentation of carbohydrates. The average final pH value of nine strains was 2·75, which is sufficient to decalcify teeth.

(3) Teeth left in contact with pure cultures over prolonged periods showed changes almost identical with those found in natural caries.

(4) Such teeth show erosion of the enamel with penetration of the dentinal tubules and the formation of liquefaction foci.

(5) The bacilli, to which we propose to give the name *B. acidophilus odontolyticus*, in their resistance to and formation of acid resemble the "acidophilus" group of Moro; biologically, however, there are several points of difference.

Finally we wish to express our indebtedness to Mr. J. Howard Mummery for the great trouble he has taken in the preparation of a number of the sections and drawings, and to Mr. J. Q. Rowett, who has generously defrayed the expenses of this research.

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ON ISOHÆMAGGLUTINATION.

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THE hypothesis put forward by von Dungern and Hirschfeldt (1911) to explain the facts of isoagglutination between human bloods is now generally accepted. According to this hypothesis, only two agglutinable factors residing in the corpuscles and two agglutinating factors residing in the serum are recognised. According as to the manner in which these factors are distributed, so is the group of any given blood determined. If the agglutinable factors resident in the corpuscles be denominated A and B respectively and the corresponding agglutinating factors resident in the serum a and b, their distribution in the various groups will be as below:

	Serum.	Corpuscles.
Group I	*Nil*	A + B
Group II	b	A
Group III	a	B
Group IV	a + b	*Nil*

If the above arrangement be the correct explanation of isohæmagglutination, it is obvious that, by saturation with corpuscles, it should be possible to remove the agglutinins corresponding to them from suitable sera. Von Dungern and Hirschfeldt do not appear to have tested their hypothesis in this way. This has been done by Koeckert (1920) with corpuscles and sera of all groups, and by Schütze (1921) for Group IV serum, in both cases with results in agreement with von Dungern and Hirschfeldt's hypothesis. The present paper is in confirmation of these findings.

TECHNIQUE.

The experiments performed consisted in the absorption of the three agglutinating sera, those of Groups II, III and IV, by the three agglutinable types of corpuscles, those of Groups I, II and III.

The sera used were, for the most part, from a day to a week old, though two of them had been kept in the ice-chest for eighteen months. Some had chloroform added, others were free from preservative; none of these factors appeared to have any substantial bearing upon the results. The corpuscles were collected in 2 per cent. sodium citrate solution in normal saline, washed

three times in normal saline and suspended in the same fluid. At first only fresh corpuscles were used, but it soon became evident that age made little difference to their activity. On standing for some days in normal saline a certain amount of laking took place, but on washing and re-suspending in fresh saline it was found that the corpuscles were as agglutinable and as actively absorbent of agglutinin as when fresh. They appeared to retain their characteristics in this respect until completely destroyed by laking.

The corpuscles were used in a 50 per cent. suspension in normal saline; for absorption five drops of this suspension were added to five drops of the serum to be tested and left standing, either overnight at room temperature, or in the incubator at 37° C. for two hours.

On completion of absorption the mixture of corpuscles and serum was shaken up and the corpuscles then thrown down by centrifuging. The supernatant fluid was pipetted off and tested as to its remaining agglutinating power. For this purpose one drop of the serum was added to one drop of a 1 in 400 suspension of the corpuscles against which it was to be tested, the two thoroughly mixed with a platinum loop and one loopful of the mixture then used to make a hanging drop preparation, which was examined under the lowest power of the microscope. The 1 in 400 dilution of the corpuscles was made by diluting a 50 per cent. suspension to the extent of 1 in 200 in the red cell pipette of a Thoma hæmacytometer. This dilution was used because it was found that with thicker corpuscular suspensions the density of the suspension tended to obscure slight degrees of agglutination. By this method the corpuscles are finally examined in a dilution of about 1 in 800, while the serum is acting in a dilution of 1 in 2; using it, it was found possible to detect agglutinins whose presence was not shown by any other means. The final reading of the hanging drop preparation was made at the end of half an hour, during which time it was occasionally agitated.

The following are examples of the experiments performed:

Experiment 1.—On Serum II (Agglutinin b).

Control: Serum II (H) *plus* Corpuscles I (Tr), *plus* Corpuscles III (Co).

Agglutination	+++	+++

(The letters in brackets denote the individuals from whom the material was obtained.)

The serum was now saturated with Corpuscles I (Tr) (Agglutinable Factors A+B), and Corpuscles III (Co) (Agglutinable Factor B).

After absorption the serum reacted—	*Plus* Corpuscles I.	*Plus* Corpuscles III.
Absorbed by Corpuscles I, Agglutination	0	0
Absorbed by Corpuscles III, Agglutination	0	0

That is to say, both Corpuscles I (A+B) and Corpuscles III (B) remove the Agglutinin b from the serum.

Experiment 2.—On Serum III (Agglutinin a).

Control: Serum III (K) *plus* Corpuscles I (Tr), *plus* Corpuscles II (W).

Agglutination	+++	+++

The serum was now saturated with Corpuscles I (Tr) (Agglutinable Factors A+B) and Corpuscles II (W) (Agglutinable Factor A).

After absorption the serum showed—	*Plus* Corpuscles I.	*Plus* Corpuscles II.
Absorbed by Corpuscles I, Agglutination	0	0
Absorbed by Corpuscles II, Agglutination	0	0

In this case both Corpuscles I (A+B) and Corpuscles II (A) removed the Agglutinin a from the serum.

Experiment 3.—On Serum IV (Agglutinins a and b).

Control:

Serum IV (D) *plus* Corps. I (Tr),	*plus* Corps. II (Re),	*plus* Corps. III (P).
Agglutination +++	+++	+++

The serum was now saturated with Corpuscles I (Tr) (Agglutinable Factors A+B), Corpuscles II (Re) (Agglutinable Factor A) and Corpuscles III (P) (Agglutinable Factor B).

After absorption the serum showed—	*Plus* Corps. I.	*Plus* Corps. II.	*Plus* Corps. III.
Absorbed by Corps. I, Agglutination	0	0	0
Absorbed by Corps. II, Agglutination	++	0	+++
Absorbed by Corps. III, Agglutination	+	++	0

In this case Corpuscles I (A+B) removed both Agglutinins a and b from the serum; Corpuscles II (A) removed a, leaving b; while Corpuscles III (B) removed b, leaving a.

These results are in conformity with von Dungern and Hirschfeldt's hypothesis.

In performing these experiments the sera used were as follows: Of Group IV, 8; of Group II, 8; and of Group III, 4. Corpuscles were used in various permutations and combinations with the sera from between two and three dozen individuals, of whom two belonged to Group I, four to Group III, and the remainder to Group II.

While making the above investigations, it was found that, though in the majority of instances the degree of saturation of the serum with the corpuscles already described was sufficient to remove all the agglutinins, this was not always the case. In the exceptions, however, the agglutinins were susceptible to being removed by a further saturation with the corpuscles. These facts pointed to differences in either the agglutinating powers of sera of the same groups, or of the agglutinable faculty of corpuscles of the same group, or of both factors.

To throw some light on this question, the relative agglutinating powers of a number of different sera were tried against the same corpuscles and, *vice versâ*, the extent to which different corpuscles were agglutinated by the same serum was investigated. The sera and corpuscles used in each individual experiment were of the same age and free from preservatives; the corpuscles were used in a 1 in 75 dilution of a 50 per cent. suspension, both in normal saline; the test was performed in small agglutination tubes, the dilutions being made by the drop method. Five drops each of the corpuscular suspension and of the serum in dilutions of 1/2, 1/5, 1/10, 1/20, 1/50 and 1/100 were thoroughly mixed in the tubes, which were then allowed to stand overnight at room temperature; the readings were taken after thorough agitation by rolling the tubes between the palms. The readings were taken by daylight and with the naked eye only, and all doubtful results were ignored.

Four grades of agglutination were recognised and recorded as follows: Conglomeration of the corpuscles into a solid mass not broken up by agitation + + +; conglomeration into a few large masses + +; a suspension of smaller masses in a clear fluid +; those cases where the fluid could not be said to be clear to the naked eye, but where, nevertheless, macroscopic masses were visible ±.

Of the tests for the relative agglutinating powers of sera from different individuals the following is an example:

			1/2.	1/5.	1/10.	1/20.	1/50.	1/100.
Corpuscles II (W) *plus*	Serum IV	(D)	+ + +	+ +	±	−	−	−
	,,	(O)	±	−	−	−	−	−
	,,	(H)	+ + +	+ +	+ +	±	−	−

These results show clearly that considerable differences exist in the titre of the a agglutinin of Group IV sera from different individuals; similar results showing differences in the titre of the b agglutinin also were obtained. It was, moreover, ascertained that, in a Group IV serum from the same individual, the relative titre of the a and b agglutinins might be very different; in some sera the titre of a and b was found to be about equal, in some a was more powerful than b, and in others the reverse was the case.

Sera belonging to Groups II and III from different individuals were also tested and found to vary greatly in their agglutinin titre. The following is an example from the Group II series:

			1/2.	1/5.	1/10.	1/20.	1/50.	1/100.
Corpuscles III (P) *plus*	Serum II	(W)	+ + +	+ +	+ +	+ +	+	±
	,,	(Re)	+	±	−	−	−	−
	,,	(Tr)	+ +	+	±	−	−	−

As with the sera, so it was found to be the case with the corpuscles, the agglutinability of those of the same group from different individuals was found to be subject to considerable variation, and, moreover, in the case of Group I corpuscles, it was ascertained that the A and B factors might be present in very different degrees. One of the Group I bloods examined gave rise to much trouble before being finally classified, for the reason that the A factor was present to such a small extent as to render the corpuscles inactive to any but a very powerful a agglutinin. This blood was first classified as Group III, and it was only the irregular behaviour of the corpuscles in absorption tests which led to further investigation and its ultimate relegation to Group I.

The following are examples of the results obtained by agglutinating corpuscles from different individuals belonging to the same group, with the same serum:

Serum IV (O) diluted		1/2.	1/5.	1/10.	1/20.	1/50.	1/100.
Plus Corpuscles II	(B)	±	−	−	−	−	−
,, ,,	(W)	+ + +	+ +	±	−	−	−
,, ,,	(Te)	+ + +	+ +	+	±	−	−

Serum II (R) diluted		1/2.	1/5.	1/10.	1/20.	1/50.	1/100.
Plus Corpuscles I	(Da)	+ + +	+ + +	+ +	+	±	−
,, ,,	(Tr)	+	±	−	−	−	−

Serum III (K) diluted	1/2.	1/5.	1/10.	1/20.	1/50.	1/100.
Plus Corpuscles I (Da)	+++	+++	++	±	−	−
„ „ (Tr)	+++	+++	+++	++	+	±

CONCLUSIONS.

I. Absorption tests performed on sera of Groups II, III and IV, using corpuscles of Groups I, II and III, confirm von Dungern and Hirschfeldt's hypothesis as to the distribution and nature of agglutinins and agglutinable factors in the four blood groups.

II. (*a*) Sera from different individuals of the same group vary in their agglutinating powers.

(*b*) The relative titre of the two agglutinins in any given Group IV serum may be equal or very unequal. Sera from different individuals of this group differ greatly in this respect.

(*c*) Corpuscles from different individuals of the same group vary as to their agglutinability by the same serum.

(*d*) The relative degree to which the agglutinable factors may be present in corpuscles from any individual belonging to Group I varies greatly; corpuscles from different individuals belonging to Group I differ in this respect.

The practical importance of the conclusions under II lies in their application to the choice of Groups II and III sera for purposes of blood grouping. For this it is necessary to have sera of a high agglutinin titre, otherwise errors, particularly in the missing of Group I cases, will occur. On this account a quantitative estimation of the agglutinin titre should be made before any serum is selected for grouping purposes, and only those sera should be used which show agglutinating power up to a dilution of at least 1/10.

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THE RELATIVE VALUE OF HUMAN AND GUINEA-PIG COMPLEMENT IN THE WASSERMANN REACTION.

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It is well known that certain active-serum methods, in which the serum to be tested provides the necessary complement, give a more delicate test in what are known as borderland cases—that is they give a positive result more readily when only a small quantity of syphilitic antibody is present. This greater delicacy is attributed to several factors acting singly or together, namely (1) the absence of the effect of heat in destroying a moiety of the specific antibody, (2) the absence of a similar effect on a non-specific anti-complementary property.

In the course of a duplicate series of tests, using an active serum method in one and the full technique with guinea-pig complement in the other, certain observations suggested another hypothesis, namely that the difference might be due to a different combining value of human complement as compared with that of the guinea-pig, with the complex syphilitic antigen plus antibody. A different combining value of various complements in this respect was noted by Noguchi and Bronfenbrenner (1911)[2], who found that among several domestic animals rabbit's complement effected a loose combination while that of the goat did not combine at all.

EXPERIMENT 1.

Experiment 1 was arranged to test the hypothesis that the difference in delicacy of an active-serum method and that of the full technique might be due to a different combining value of the complements employed. For this purpose a triplicate series of tests was performed on several batches of sera as they came in from the hospital and venereal disease clinics.

Series A. Active serum method.
„ B. } Inactivated serum { Full technique with a foreign human complement.
„ C. } Inactivated serum { Full technique with guinea-pig complement.

As far as possible Series A was made to correspond, in volumes and concentration of materials used, with Series B and C. This correspondence was made practicable by the fact that the volume of serum tested (inactivated as in the full technique) contains, when active as in the active-serum method,

complement equivalent to about 3 minimum hæmolytic doses (m.h.d.) for the volume of sensitised sheep's red cells used in each of the series of tests. A certain percentage of sera are unsuitable with the active serum method owing to absence of natural complement. Fleming's (1909) technique was employed for Series A, but modified to bring the correspondence to as great a degree as possible. Fleming's reaction, a three-tube method, allows of readings of degrees of positivity by varying the strength of the antigen in the second and third tubes. The highest strength of antigen used in Series A corresponded to the strength used throughout in Series B and C, in which an ordinary three-tube method (with varying doses of complement) was employed, *i. e.* Tube 1 contained inactivated test serum, 3 titrated m.h.d. of complement and saline diluent; tubes 2 and 3 contained inactivated test serum, 3 and 5 m.h.d. of complement respectively and antigen. They were incubated for the usual period and fully sensitised sheep's red cells subsequently added to all the tubes and the readings taken in the usual manner. The only difference between Series B and C was the origin of the complement—in B, man, and in C, the guinea-pig.

Series B and C were put up side by side. Series A was put up, as is necessary when using active serum, within 24 hours of collection and in smaller batches. Upwards of 100 sera in several batches spread out over several months were tested in this manner—a number sufficient to bring out any well-marked difference in the readings of the three methods. It is obvious that any accurate quantitative comparison of Series A with B and C is impossible. In A the complement is not titrated and the result is given in terms of antigen fixed instead of in doses of complement fixed, and considerable experience is necessary in making the readings, especially with weakly positive sera. The readings given by Series A were, however, found to tally in a remarkable manner with those given by Series B; such slight variations as were noted could more readily be accounted for by the other variations of the two methods than by the fact that the serum was inactivated in B. A tabulation of the results is not necessary; for practical purposes they were the same. The difference between the readings of B and C was from the first a very striking one, and the experiment using Series B and C in parallel was continued for several months, upwards of 600 sera being tested. In the first part of these comparisons human complement was derived from one individual only; in the later tests several human complements were included as controls to the original in multiple parallel series, these tests being the first part of Experiment 2, which will be outlined later, Experiments 1 and 2 thus overlapping. Pooled guinea-pig sera were employed, as a general rule from three animals.

The results in terms of positive and negative were as follows:

Number of tests	610
Positive with human complement	208
Positive with guinea-pig complement	171

This balance of 37 in favour of fixation with human complement occurred among the borderland cases, primary, tertiary and treated syphilis. In considering the discordant results the following signs of hæmolysis or its absence may be employed: +, +, + denotes complete hæmolysis in all three

tubes, the first sign indicating the control and the second and third the tubes with antigen and 3 and 5 m.h.d. of complement; +, −, − indicates complete absence of hæmolysis in the antigen tubes or a strong positive; ± may be taken to indicate degrees of hæmolysis, and so +, −, ± will represent a weak positive and +, ±, + a doubtful one. Now the method of testing allows of the detection of a difference of not more than of 5 m.h.d.; such a discrepancy has not infrequently been noted, a +, −, − with human complement giving a +, +, + with guinea-pig complement. As a rule the discrepancy might be accounted for by a different fixing value of from 2 to 4 m.h.d., a reading of +, −, − being converted into +, −, +, or +, ±, +, and a reading of +, −, ± into +, ±, + or +, +, +. In no duplicate series was there a closer approximation. An occasional doubtful positive +, ±, + with guinea-pig complement, in which the anti-complementary factor was evidenced by a prolonged delay in the completion of hæmolysis in the control tube, gave a +, +, + with human complement. It may be noted that throughout the experiment the anti-complementary factor to guinea-pig complement was more in evidence than that to human complement. One very exceptional serum from a secondary syphilitic required 12 m.h.d. of human complement to hæmolyse the control tube; 24 m.h.d. of guinea-pig complement was insufficient.

In the experiment so far recorded the evidence in favour of greater fixation with human complement is derived from readings with weekly positive sera. In the following table is shown the result of titrating the strength of a strongly positive serum by dilution of the three essential ingredients.

TABLE I.—*Quantitative Estimates of the Reacting Power of a Syphilitic Serum Using Human and Guinea-pig Complement.*

(*a*) By dilution of complement. Serum (1 in 5) and antigen (1·5 per cent.) constant.

Control 3	6	7	8	9	10	11	12	13	14	m.h.d. complement.
+	−	−	−	−	±	±	±	±	+	human.
+	−	±	±	±	±	+	+	+	+	guinea-pig.

(*b*) By dilution of antibody serum (preliminary dilution 1 in 20 instead of the usual 1 in 5). Three m.h.d. complement and antigen (1·5 per cent.) constant.

60	50	40	30	20	10	5	c.mm. serum.
−	−	−	−	±	±	+	human complement.
−	±	±	+	+	+	+	guinea-pig complement.

(*c*) By dilution of antigen. Three m.h.d. complement and serum (1 in 5) constant.

1·5	·75	·37	·18	·09	antigen per cent. total volume.
−	−	−	±	+	human complement.
−	±	+	+	+	guinea-pig complement.

Comparative hæmolytic titre of the undiluted complements. Guinea-pig : human :: 5 : 1 in the preliminary titration.

+ represents complete hæmolysis; ± gradations from commencing to almost complete; − absence of hæmolysis.

So far these experiments show that in the technique used the homologous complement is more readily fixed than that of the guinea-pig in the combination with syphilitic antigen and antibody, and that the active serum methods may owe their greater delicacy to this more ready fixation rather than to the absence of inactivation of the serum tested. The delicacy of the reaction is, however, of little importance if it does not permit control of the variation in the capacity for fixation of the complements used. Various workers have pointed out a serious defect in the use of guinea-pig complement, which is sufficient to negative any attempts to control the test except with a most elaborate and exclusive technique. This defect is the absence of any ratio between the hæmolytic titre of a complement and its capacity of being fixed in the combination with syphilitic antigen and antibody. There is a very great difference among various guinea-pigs in this respect. Three authorities as to this point may be quoted:

Browning and Kennaway (1919) say "the complement is an important source of variation, since, as was pointed out by Browning and Mackenzie, there is no constant relationship between its hæmolytic power and its capacity for being bound by the mixture of antigen and antibody." "The same positive serum may require from 5 to 16 doses to produce commencing lysis,"—and, "These variations are quite irregular, and depend on factors which cannot so far be rendered constant."

Noguchi and Bronfenbrenner (1911)[1] put up simultaneously 41 complements from 41 guinea-pigs with a syphilitic serum and estimated the amount of complement removed in hæmolytic units. Their figures are:

0 units	1 specimen =	2·4 per cent.
1·5 to 3·5	8 specimens = 19·5	,,
4 to 5	20 specimens = 48·7	,,
5·5 to 9·5	12 specimens = 29·2	,,

There is here an ample range—a range somewhat similar to that observed by Browning if the exceptional 2·4 per cent. specimen is omitted—for the disappearance of many positive reactions.

Griffith and Scott (1920) say "of all the reagents in the Wassermann test complement is the least capable of standardisation, and is certainly the reagent which is most often responsible for the discrepancies on different occasions."

Browning uses ox blood, Noguchi human and Griffith and Scott sheep's blood with their appropriate amboceptors as indicator antigen.

EXPERIMENT 2.

Experiment 2 consisted of testing 44 human complements from 44 individuals to see if the hæmolytic titre bore any relation to the fixing capacity in the presence of syphilitic antigen and antibody, and to determine whether human complements differed from one another in fixing power in a fashion similar to that observed amongst various guinea-pigs by other workers. The tests were done in several batches spread out over several months, and every batch included Complement No. 1 as a control to an unknown complement both as to hæmolytic titre and what may be called syphilitic titre.

Complement No. 1 has been used for several years, and has never been known to vary in the tests from week to week in its hæmolytic titre or in the readings with the many weak positives used as controls in hundreds of batches of tests. The hæmolytic titre was determined with and without antigen for the full period of incubation in the water bath before the addition of sensitised corpuscles. Subsequently to this, duplicate or multiple parallel series of Wassermann reactions were put up with 3 and 5 m.h.d. of the complements and one or more inactivated weakly positive syphilitic sera giving reactions +, −, ±. Of the 43 complements tested against No. 1, 41 resembled it so closely as to show at times only varying degrees of hæmolysis in tube 3, such variations as would be accounted for by small differences inherent in the method of standardisation of complement or perhaps in differences of an anti-complementary factor in the active complement-containing serum—a variation which could be expressed in a fraction of a m.h.d. Two complements only, one from a laboratory assistant with phthisis (the result with this one was repeated after an interval of one year), and another from a patient with an extensive boil on the neck, converted a +, −, ± to +, ±, +, neither of them varying sufficiently to convert a weak positive into a complete negative—a variation in fixing power of 2 m.h.d. Whether or not the illness of these two accounted for a deficient fixing power is not known. The other complements were derived from apparently healthy individuals of ages from 60 to 3 years, mostly males.

The results of the comparative tests showed that for practical purposes with all the healthy human complements used there is a constant ratio between the hæmolytic titre and the syphilitic titre in the Wassermann reaction, and that the fixing power of various human complements does not vary in a manner similar to that observed amongst guinea-pigs.

A few points with regard to the hæmolytic titre of the human complements may be mentioned here. Complement No. 1 (the standard) in the technique employed contains 3 m.h.d. in 10 c.mm.; two human sera contained 2 m.h.d. in 10 c.mm., four contained 4 m.h.d. in the same quantity; the others were identical with Complement No. 1 within the limits of the method of standardisation. The variations in quantities of natural amboceptor for sheep's cells has not been taken into account; this would appear from these and other experiments, not included in this paper, to be of not much account—at any rate with this technique. The human complements were all titrated and used on the day of collection. The tests of the titration with and without antigen of all the complementary sera were most satisfactory; several were tested both without and with the full incubation in the water bath and showed that very little available complement was used up during the incubation. This incubation is of course necessary in view of the possibility of a serum being syphilitic. They all showed that a clear 2 m.h.d. of complement was available for the final tests. It has never been necessary when doing tests with human complement to cease work and look for fresh material, and the completion of the task in hand within the hours allotted to it is always assured, and with the knowledge that a result obtained with the controls of the previous batch of tests will be repeated with certainty with subsequent batches.

It is not proposed to detail in this paper the technique employed. In

relative volumes of materials used and relative periods of incubation it does not differ from other methods. Some essential details are :

(1) The diluent salt solution.
(2) Quantities of materials.
(3) Antigen.

(1) *The diluent salt solution.*—Too much emphasis cannot be laid on this apparently trifling detail. In the early days of working with active-serum methods it was noticed on several occasions that there was an absence of hæmolysis with all or an unusually large percentage of the human sera to be tested. This was traced to absorption of complement by the distilled water used in making up the salt solution. On using freshly distilled water of which the first fraction had been discarded this trouble disappeared, and all the sera were found to give satisfactory hæmolysis. Human complement is of low titre, and it is essential that none of it should disappear in combination with the diluents used in the reaction. The salt solution used in these tests has been made up in a two-litre flask of alkali-free glass with freshly distilled water and the whole thoroughly dissolved and shaken and then autoclaved at 110°C. The flask, being fitted with a hooded pipette and tap, serves for several batches of tests and remains sterile with due care.

(2) *Quantities of materials used.*—Total quantity in each tube = 0·2 c.c., comprising—

Diluted complement 50 c.mm. = 10 c.mm. to 16·6 c.mm. serum.
Diluted test serum 50 c.mm. = 10 c.mm. serum.
Antigen or salt solution 50 c.mm.
3 per cent. sensitised sheep's red cells 50 c.mm. (sensitised with 5 m.h.d. amboceptor).

The amount of human blood necessary to provide complement for titration and the test of 25 to 30 inactivated sera is 3 c.c. The technique in manipulating these small quantities is that of Wright (1921).

(3) *The antigen.*—Both ethyl alcohol and cholesterol are very anti-complementary in the reaction with human complement, and an antigen devised by Fleming and used for the last seven years in this laboratory has been used in these tests. It is free from cholesterol, and its high concentration in the necessary constituents of human heart muscle made possible by the removal of the watery moiety allows of a greater dilution of the alcoholic solvent. It is very simply made up. 100 grm. human heart free from fat is minced and put into a flask with 500 c.c. acetone. After 24 hours at room temperature the solid portion is filtered off and dried in the incubator. This is then put into another 500 c.c. acetone and treated a second time in the same manner. The dried material is then extracted for a week on the bench in 100 c.c. absolute alcohol, and the clear amber-coloured fluid is filtered off and stored for use. Alternatively the dried material may be stored for future extraction and keeps well. A 10 per cent. dilution in saline of this extract should be "crystal clear." Should a marked turbidity result on dilution, as has happened with some samples, the material should be discarded : turbid extracts are more anti-complementary than is desirable. The antigen volume used in the test is as a rule a 7 per cent. dilution—or 1·75 per cent. of the total volume.

CONCLUSIONS.

(1) Among healthy human complements there is for practical purposes a constant ratio between the hæmolytic titre and the capacity for being fixed with syphilitic antigen and antibody. This constant ratio enables the reaction to be standardised, and in this respect human complement is superior to that of the guinea-pig in the Wassermann reaction.

(2) The fixation of human complement in the reaction is much more complete than that of the guinea-pig and allows of a more delicate test without the use of cholesterinised antigen.

(3) The difference in delicacy observed in the active-serum methods is probably due to this greater fixation of human complement, and not to the absence of inactivation of the serum to be tested.

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FURTHER EXPERIMENTAL STUDIES ON IMMUNISATION AGAINST *B. DYSENTERIÆ* (SHIGA) AND ITS TOXINS.

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In a previous communication (Kanai, 1921) experimental evidence has been brought forward to show that rabbits immunised by the subcutaneous administration of a carbolised vaccine of *B. dysenteriæ* (Shiga) acquired a more solid immunity to living Shiga bacilli intravenously administered than those immunised *per os*. In the course of this work it was noted that those animals which succumbed to the test inoculation invariably showed some paralysis prior to death, with the constant post-mortem finding of hæmorrhages in the medulla and spinal cord, whereas any pathological change in the intestinal mucosa apart from œdema was of rare occurrence. This mode of reaction on the part of the rabbit to dysentery toxin is well known, and the difference in behaviour of this animal and the guinea-pig to the toxin of *B. dysenteriæ* has led to the conclusion that there exist in reality two toxins. The one, especially toxic for the rabbit, gives rise to the paralytic symptoms, the other produces pyrexia and general marasmus and is more readily demonstrated in the guinea-pig. These two toxins have been variously referred to as "rabbit toxin" and "guinea-pig toxin," or "paretische Gift" and "marantische Gift" (Pfeiffer, 1908), and attempts to establish the nature of these toxins have been productive of much discussion. It would be impossible in the space of a short article to review the literature on this question, but suffice it to say that up till quite recently it remained undecided. The recent researches of Olitsky and Kligler (1920), however, would appear to offer a solution of this question in so far as the effect on the rabbit is concerned. These workers claim to have demonstrated unequivocally the presence of two types of dysentery toxin. One is soluble, a true exotoxin, is destroyed by heating at 80° C., and is responsible for the paralytic symptoms in the rabbit. It is essentially a neurotoxin and gives rise to those hæmorrhages in the medulla and cord to which reference has already been made. The other type they classify with the so-called endotoxins. It is more heat-stable than the neurotoxins, withstands a temperature of 80° C., and produces the hæmorrhages in the intestinal mucosa which determine the characteristic lesion of dysentery.

Besredka (1919) claims, in his recent publication on immunisation by the oral route, that the immunity produced in animals by this method to *B. dysenteriæ* (Shiga) is a local immunity. The cells of the intestinal mucosa have acquired a new character; they are immune to dysentery toxin. Was it

possible, then, that those rabbits which in our last study had been immunised *per os* and which were unable to withstand the test-dose of living bacilli, succumbed to the neurotoxin (exotoxin) alone, and that they were immune to the so-called endotoxin, which, according to Olitsky, produces the hæmorrhagic lesions in the intestinal wall? It may be recalled that these animals showed lesions in the central nervous system only. It was decided to test the correctness of this hypothesis.

OUTLINE OF THE PROPOSED EXPERIMENTS.

It is well known to all who have studied the toxicity of dysentery bacilli that strains of the same organism differ markedly in their power to produce intestinal lesions in the rabbit. It was proposed first of all, therefore, to see if a strain of *B. dysenteriæ* (Shiga) could be found which produced chiefly intestinal lesions. It was thought that with such a strain it would be possible to test the local immunity produced by the oral administration of the vaccine.

TABLE I.

Rabbit.	No. of strain.	Symptoms.	Dysentery bacilli recovered from—	Post-mortem findings.
No. 60. 1400 gr.	151.	Paralysed on 1st day, died 28 hours after injection.	Gall-bladder, spleen and kidney.	*Lung*: Hæmorrhages. *Small intestine*: Injection of mucous membrane and leucocytic infiltrations of tunica propria.
No. 61. 1870 gr.	152.	Found dead 19 hours after injection.	Spleen.	No hæmorrhages in lung, otherwise changes similar to those in No. 60 were observed.
No. 62. 1630 gr.	208.	Paralysed on 3rd day. Died on the 5th day. No diarrhœa.	—	*Small intestine*: Hyperæmia and œdema. *Appendix* shows s milar changes. *Large intestine* appears normal.
No. 63. 1580 gr.	753.	Paralysed on 1st day, died on the 2nd day, no diarrhœa.	Gall-bladder, appendix and lung.	*Lung*: Hæmorrhages. *Liver*: Congested. *Small intestine and duodenum*: Some œdema and hyperæmia.
No. 64. 1330 gr.	752.	Found dead next morning.	Gall-bladder, spleen and kidney.	Same as in Rabbit 63.
No. 65. 1840 gr.	754.	Paralysed on 2nd day, died on 3rd day.	Gall-bladder and spleen.	No hæmorrhages in lung or noteworthy changes in gut-wall.
No. 78. 1500 gr.	Besredkas' strain, $\frac{1}{2}$ of an agar slope.	No paralysis and no diarrhœa. Rabbit found dead on the 2nd day.	Gall-bladder and spleen.	*Lung*: Small hæmorrhages. *Liver and kidney*: Congested. *Small intestine*: Œdema and injection of submucosa. *Large intestine*: Œdema and injection with some hæmorrhages in submucosa and tunica propria.
No. 77. 1380 gr.	The above strain, $\frac{1}{5}$ of an agar slope (weak growth).	No symptoms; survived.		

Further, the preparation of dysentery exo- and endotoxin, on the lines of Olitsky's work, was envisaged, with the object of testing the relative immunity of rabbits, immunised subcutaneously and orally, to these two toxins.

COMPARISON OF THE TOXICITY OF VARIOUS STRAINS OF *B. DYSENTERIÆ* (SHIGA).

Seven strains of *B. dysenteriæ* (Shiga) were taken and injected intravenously into rabbits in a dose of one-fifth of a 24-hour agar slope culture. This quantity of bacilli was suspended in 1·0 c.c. of normal saline. Those rabbits which died as the result of the inoculation of the living bacilli were examined for the presence of hæmorrhagic lesions—particularly in the intestine—while the gall-bladder, heart-blood, spleen, kidney, lung and liver were submitted to cultural investigation for the presence of dysentery bacilli. The results of this experiment are given in Table I.

Of the seven strains employed in this experiment Besredka's alone appears capable of producing hæmorrhages in the intestine, but even with this strain they were of a trivial character. It was decided, therefore, to proceed with the preparation of exo- and endotoxin with which to test the local immunity of the immunised rabbits.

PREPARATION AND CHARACTERS OF DYSENTERY TOXINS.

Exotoxin.

The growths from fifteen 24-hour agar slopes (strain No. 151) were suspended in 10·0 c.c. normal saline and immediately filtered through a Berkefeld filter-candle (N). The filtrate was tested on rabbits by intravenous inoculation and the results obtained are recorded below (Table II).

TABLE II.

Rabbit.	Body-weight.	Dose injected.	Symptoms.	Post-mortem findings.
No. 88.	1400 grs.	5·0 c.c.	Paralysis of fore-legs 1st day after inoculation. No diarrhœa. Found dead on 2nd day.	*Lungs, liver and kidneys* are congested. *Spleen* congested, Malpighian bodies swollen, and show some small hæmorrhages. *Large intestine*: Œdema and injection of submucosa and tunica propria, with some extravasation of red cells in the œdematous portions. *Small intestine*: Injection and œdema of submucosa. *Medulla and spinal cord.* Pia œdematous and vessels dilated. Some hæmorrhages in the grey substance and chromatolysis of nerve-cells.
No. 76.	2330 grs.	3·0 c.c.	Dyspnœa 2 hours after injection lasting 2 hours. Hind legs paralysed next morning. Died 28 hours after inoculation.	Similar to those observed in No. 88.
No. 77.	2230 grs.	1·0 c.c.	Hind legs paralysed on 2nd day. Died 3 days after injection.	—

It will be seen from this experiment that when a 24-hour agar culture of *B. dysenteriæ* (Shiga) is suspended in normal saline and immediately freed from the bacillary bodies by filtration, the filtrate is highly toxic for rabbits, producing the same symptoms and post-mortem changes as the whole bacilli.

Resistance of "exo-toxin" to heat.—Exotoxin, prepared as in the previous experiment, was submitted to temperatures of 60° C. and 80° C. for one hour and inoculated intravenously into rabbits in doses of 1·0 c.c., with the following results:

TABLE III.

Rabbit.	Body-weight.	Toxin.	Results.
No. 83.	1320 grs.	1·0 c.c. "exotoxin" heated at 80° C.	No symptoms. Survived.
No. 84	1100 grs.	1·0 c.c. "exotoxin" heated at 60° C.	Paralysis 1st day. Found dead on morning of 2nd day.

Post-mortem findings in Rabbit No. 84:—*Lungs:* Congestion and hæmorrhages. *Spleen* and *kidney:* Congested. *Liver:* Congested—shows evidence of infection with coccidia. *Small intestine:* Injected. Polynuclear infiltration of submucosa, with some hæmorrhages. *Large intestine, appendix:* Normal except for presence of coccidia.

It is seen, then, from this experiment that the readily soluble portion of the dysentery toxins (exotoxin) is rendered non-toxic for the rabbit by heating it at 80° C. for one hour, whereas a temperature of 60° C. leaves it capable of producing both lesions in the central nervous system and hæmorrhage in the intestinal mucosa.

Minimum lethal dose of "exotoxin."—An attempt to determine the M.L.D. of this exotoxin gave inconclusive results. Three rabbits were inoculated intravenously with 0·1 c.c., 0·5 c.c. and 1 c.c. respectively. The two which received 0·1 c.c. and 1·0 c.c. survived, having shown no symptoms whatever, whereas the third with 0·5 c.c. became paralysed on the first day, recovering on the fourth day after inoculation.

Endotoxin.

It was held by Klein (1907) and Heller (1909) that the toxin of *B. dysenteriæ* (Shiga) was of the nature of an endotoxin, but, as already stated, the experiments of Olitsky and Kligler would appear to demonstrate the existence of two dysentery toxins, the one soluble, destroyed at 80° C.—neurotoxin—the other remaining attached to the bacillary bodies, resisting a temperature of 80° C. and giving rise to the hæmorrhagic lesions in the intestine. The following experiments were undertaken to determine the toxicity of dysentery bacilli after removal of the soluble toxin by washing in normal saline. The strains employed were No. 151 and Besredka's.

Toxicity of bacilli washed in saline five times.—The growth obtained from fifteen 24-hour agar slopes was suspended in 5·0 c.c. normal saline and

centrifuged to throw down the bacilli. The clear supernatant fluid was pipetted off and an additional 5·0 c.c. saline added, in which the deposit was re-suspended. This was repeated five times. The final suspension was heated at 60° C. for one hour, centrifuged again, and the toxicity of the supernatant fluid and the bacillary deposit tested on rabbits (Table IV).

TABLE IV.

Rabbit.	Body-weight.	Inoculum.	Result.
No. 92.	1500 grs.	500×10^6 washed bacilli.	Survived.
No. 90.	1520 grs.	1000×10^6 washed bacilli.	Hind legs paralysed 2nd day. Killed 3rd day for examination.
No. 91.	1300 grs.	2000×10^6 washed bacilli.	Forelegs paralysed 1st day. Died 2nd day.
No. 93.	1550 grs.	3·0 c.c. supernatant.	Died 2nd day.

Post-mortem findings in rabbit No. 90:—*Lungs:* Congested. *Spleen:* Hæmorrhages in pulp, and some necrosis of the cells in the germ centres. *Liver:* Congested. *Small intestine:* Vessels of submucosa dilated and some œdema of the tunica propria. *Large intestine:* Œdema. Some small hæmorrhages in tunica propria. *Spinal cord:* Numerous small hæmorrhages in grey matter and chromatolysis of nerve-cells.

Rabbit No. 93 showed very similar changes.

Effect of a temperature of 80° C. on endotoxin.—The same bacterial emulsion, washed five times (as used in the last experiment), was heated at 80° C. for one hour and three rabbits inoculated intravenously with varying doses. The result of this experiment is given in Table V.

TABLE V.

Rabbit.	Body-weight.	Dose inoculated.	Result.
No. 94.	1210 grs.	2000×10^6	Survived. No symptoms.
No. 95.	1300 grs.	1000×10^6	,, ,,
No. 96.	1880 grs.	4000×10^6	,, ,,

Rabbit No. 96 was killed and examined, the post-mortem findings being as follows:—*Lungs:* Congested. *Spleen:* Pulp congested and shows hæmorrhages. *Kidney:* Hæmorrhages in cortex. *Small intestine:* Injection of vessels of villi and œdema of tunica propria. *Large intestine:* Injection of vessels of tunica propria. Some small hæmorrhages in wall of *appendix*. *Spinal cord* and *medulla:* No change.

This experiment was repeated, but a suspension of unwashed bacilli was employed in place of bacilli washed five times in normal saline. The suspension was heated for one hour at 80° C. as before.

TABLE VI.

Rabbit.	Body-weight.	Dose inoculated.	Result.
No. 132.	1920 grs.	5000×10^6.	No symptoms.
No. 131.	1900 grs.	$10,000 \times 10^6$.	„
No. 135.	2010 grs.	$20,000 \times 10^6$.	Diarrhœa. No paralysis. Found dead 1st day after inoculation.
No. 136.	2040 grs.	$40,000 \times 10^6$.	Weak 1st day after inoculation. Recovered.

Post-mortem findings in Rabbit 135:—*Lungs*: Hæmorrhages in right lobe. *Spleen*: Severe congestion of pulp and small hæmorrhages in folliculi. *Liver*: Hæmorrhages. Necrosis of parenchyma with leucocytic infiltration of necrotic areas. *Kidneys*: Congestive swelling of convoluted tubules. *Small intestine*: Hyperæmia of tips of villi. *Stomach*: Hæmorrhages in submucosa. *Large intestine*: Œdema and hæmorrhages in tunica propria (heavily infected with coccidia). *Spinal cord*: Small capillary hæmorrhages in grey matter of cervical portion and in medulla.

From the above experiments it would appear that the readily soluble portion of the toxin of dysentery bacilli, the so-called exotoxin, produces changes chiefly in the central nervous system. These are of the nature of capillary hæmorrhages in the grey matter of the cord and medulla and chromatolysis of the nerve-cells. The action of this exotoxin is not confined to the nervous system, however, as a general action on the circulatory system is evinced by capillary hæmorrhages throughout the body. Thus hæmorrhages were noted in the lungs, liver, kidney, spleen, and especially in the submucosa of intestine, which invariably showed some œdema accompanied as a rule by extravasation of red cells. Heating for one hour at 60° C. did not reduce the toxicity of this soluble portion of the dysentery toxin.

From the experiments carried out with the washed bacilli it is evident that a large proportion of the toxin still remains closely united with the bacterial bodies, even after they have been washed five times and extracted with normal saline for one hour at a temperature of 60° C. Bacilli so treated were still highly toxic for rabbits, producing much the same changes as those arising from the inoculation of the soluble portion of the dysentery toxin. When these washed and extracted bacilli are submitted to a temperature of 80° C. for one hour their toxicity for the rabbit is greatly reduced. Of the three rabbits inoculated with bacilli so treated none died or showed any symptoms whatever. The findings in one of these three rabbits, which was killed and examined, showed complete absence of change in the central nervous system, though there were hæmorrhages in the various viscera. These experiments were carried out with strain No. 151, and it must be admitted that the attempts to prepare an exo- and endotoxin from it were not attended with any great success, though bacilli when heated at 80° C. seemed to have lost to a certain extent their neurotoxin and were yet capable of giving rise to hæmorrhages, particularly in the intestinal submucosa. The following experiment was therefore carried out with Besredka's strain, to see whether it would be possible to achieve with another strain of *B. dysenteriæ* (Shiga) what had

proved unsuccessful with strain 151, viz. the separation of exotoxin from endotoxin. This experiment is summarised in Table VII.

TABLE VII.

Rabbit.	Material inoculated.	Result.
No. 123.	Fifth washing of the growth from ten agar slopes. (Bacilli washed each time with 5·0 c.c. saline, fifth washing filtered through a Berkefeld filter N.)	Paralysis of forelegs on 1st day. Died 2 days after inoculation.
No. 124.	The washed bacilli from above, suspended in saline, heated at 60°C. for one hour and centrifuged. Bacilli so obtained inoculated intravenously in a dose of 2000×10^6.	Paralysed and died the 1st day after inoculation.
No. 126.	The tenth washing of five agar slope cultures. (Bacilli washed each time with 5·0 c.c. saline, the tenth washing filtered through a Berkefeld filter N.)	Survived. No symptoms.
No. 127.	The bacilli after tenth washing, heated for one hour at 60°C., washed three times with normal saline (5·0 c.c. saline on each occasion). Bacilli so treated inoculated intravenously in a dose of 3000×10^6.	No paralysis. No diarrhœa. Died suddenly on 3rd day.
No. 125.	Bacilli as prepared for inoculation of Rabbit No. 127 heated for one hour at 80°C. and inoculated intravenously in a dose of 4000×10^6.	Survived. No symptoms.
No. 128.	Twenty-four hour growth on agar suspended in normal saline, heated for one hour at 80°C. and inoculated intravenously in a dose of $10{,}000 \times 10^6$.	No diarrhœa. No paralysis. Died 1st day after inoculation.

The post-mortem examination of those rabbits which succumbed revealed precisely the same changes as those produced by similar preparations made with strain No. 151.

It will be seen, then, from the above experiment that the attempt to separate endo- and exotoxin from Besredka's strain of *B. dysenteriæ* (Shiga) had not been attended with any more success than in the case of strain No. 151. The experiments, however, demonstrate the fact that when a 24-hour culture of *B. dysenteriæ* (Shiga) is suspended in normal saline and the bacilli then removed by centrifugalisation and filtration, the filtrate is highly toxic for the rabbit, *giving rise to similar changes to those produced by the whole bacilli.* Both these two fractions, the supernatant fluid and the washed bacilli, appear to contain the two toxins, the neurotoxin and the hæmorrhagic toxin, but by heating the washed bacilli for one hour at 80° C. their power of producing lesions in the central nervous system is destroyed. They are still capable, however, of giving rise to hæmorrhages in the various viscera when inoculated intravenously, these changes occurring constantly in the submucosa of the intestine. A temperature of 60° C. is without effect on the toxicity of these fractions of dysentery toxin.

RESISTANCE OF IMMUNISED RABBITS TO TOXIN FRACTIONS.

It now remained to test the immunity of rabbits, immunised orally and subcutaneously, to these two fractions of dysentery toxin. Before doing this,

however, it was decided to see if any pathological changes occurred in rabbits as the result of the administration of large doses of dysentery vaccine *per os*. Five rabbits were taken and to each was given one-fifth of a Roux-bottle culture of *B. dysenteriæ* (Shiga), killed by heating for one hour at 60° C. These rabbits were killed at varying intervals after the ingestion of vaccine and the post-mortem finding are given below (Table VIII).

TABLE VIII.

Rabbit.	Time of killing.	Post-mortem findings.
No. 97 (1325 grs.).	4 hrs. after ingestion.	Some congestion of appendix, otherwise normal.
No. 99 (1750 grs.).	24 hrs. „ „	Congestion of appendix, and tips of villi in small intestine. Large intestine showed presence of coccidia.
No. 100 (1850 grs.).	48 hrs. „ „	*Spleen* enlarged, congested. Shows some nodular lesions. *Liver* shows nodular lesions. *Kidney* hæmorrhages.
No. 101 (1660 grs.).	96 hrs. „ „	*Small intestine*: Congestion of submucosa with some petechial hæmorrhages. *Appendix*: Congestion and eosinophile infiltration—coccidia. *Kidney*: Congestion. *Liver*: Hæmorrhages. *Spinal cord*: Capillary hæmorrhages in cervical portion.
No. 98 (1550 grs.).	2 weeks „ „	*Small intestine*: Congestion. *Large intestine*: One or two hæmorrhages. Coccidia. *Appendix*: Congestion and œdema of tunica propria. *Kidneys*: Hæmorrhages, with accompanying degenerative changes in parenchyma. *Spinal cord and medulla*: Congestion and capillary hæmorrhage.

Unfortunately, four out of the five rabbits were infected with coccidiosis, which detracted to a great extent from the value of this control experiment, as attention has frequently been drawn to the production of hæmorrhages in rabbits by this parasite. However, the experiment is not without interest. Undoubtedly some of the hæmorrhages were due to coccidiosis, but in rabbits Nos. 101 and 98 these occurred in kidney and central nervous system, where no parasites could be found. It seems legitimate to conclude, therefore, that these latter hæmorrhages were the result of the ingestion of dysentery vaccine, the dysentery toxin having passed through the intestinal wall into the general circulation.

Six rabbits were immunised by the oral administration of a Shiga vaccine (strain No. 151, killed at 60° C.), the dose employed being one-fifth of a Roux-bottle culture, and three doses being given at 10 days' interval. Another series of six rabbits received 3 doses of a carbolised vaccine of the same strain inoculated subcutaneously, the interval between the injections being again 10 days. The doses employed were: 1st dose, 50×10^6; 2nd dose, 100×10^6; 3rd dose, 200×10^6.

Ten days after the last immunising dose four rabbits in each series were given varying doses of the soluble dysentery toxin intravenously, whilst the remaining two animals in each series received intravenous doses of washed dysentery bacilli which had been submitted to a temperature of 80° C.

The dose of washed bacilli (heated to 80° C.) was fixed at 20,000 × 10^6 (from result of experiment detailed in Table VI), but as this proved insufficient to kill the control, Table IX (annexed) gives no information as to immunity to toxic substances present in the washed and heated bacilli.

TABLE IX.

A.—Rabbits Immunised by Subcutaneous Route.

Rabbit.	Weight.	Test-dose.	Result.
No. 102.	1350 grs.	0·7 c.c. "soluble" toxin intravenously.	Paralysed on 2nd day. Recovered on 4th day. No further symptoms. Killed and examined. *Lung*: Congestion and hæmorrhages. *Spleen*: Severe congestion and hæmorrhages in pulp. *Liver*: Small hæmorrhages. *Small intestine*: Congestion and œdema of submucosa. *Spinal cord and medulla*: Hæmorrhages in grey substance.
No. 103.	1650 grs.	0·7 c.c. "soluble" toxin intravenously.	Paralysed on 2nd day. Died on 4th day. *Lung*: Congestion and serous exudate in alveoli. *Spleen*: Malpighian bodies show hæmorrhages and œdema of germ centres. *Small intestine*: Injection of submucosa. *Large intestine*: Injection of submucosa. *Appendix*: Eosinophile infiltrate. Coccidiosis. *Spinal cord*: Vessels of grey substance injected—some hæmorrhages. Nerve-cells show chromatolysis and some vacuolisation. *Medulla*: Injection of vessels and hæmorrhages in grey substance.
No. 104.	1150 grs.	1·4 c.c. "soluble" toxin intravenously.	Hind legs became paralysed 20 hours after inoculation. Died soon after. *Lung*: Severe congestion and hæmorrhage. *Spleen*: Congestion and hæmorrhage. *Liver*: Congestion and hæmorrhage, some fibrosis and infiltration with eosinophile leucocytes. *Small intestine*: Submucosa shows injection of vessels, with small hæmorrhages. *Large intestine*: Tunica propria shows injection of vessels, œdema, eosinophilic infiltrate and many small hæmorrhages. *Spinal cord, medulla*: Hæmorrhages and degenerative changes in nerve-cells.
No. 105.	1600 grs.	Ditto.	No symptoms. Survived. Killed and examined. *Lung, liver and spleen*: Congestion. *Small intestine*: Œdema. Injection of vessels and hæmorrhages in tunica propria. *Large intestine*: Injection and œdema of submucosa. *Spinal cord and medulla*: *Nil.* Rabbit infected with coccidia.
No. 106.	2150 grs.	20,000 × 10^6 washed bacilli killed by heating at 80° C. for one hour.	No symptoms. Survived. Killed and examined. *Lungs*: *Nil.* *Spleen*: Enlarged. Swelling of cells in germ centres. *Small intestine*: Small macroscopic hæmorrhages. *Large intestine*: Many small hæmorrhages and œdema of tunica propria. *Spinal cord*: Injection of vessels in grey matter. *Medulla*: *Nil.*
No. 107.	2000 grs.	Ditto.	No diarrhœa. No paralysis observed. Found dead on morning of 3rd day. *Lung*: Œdema and fibrinous exudate in alveoli. *Liver and kidney*: Congested. *Small intestine*: Injection of submucosa. *Large intestine*: *Nil.* *Spinal cord*: Large hæmorrhages in grey matter of cervical and lumbar regions. Nerve-cells show degenerative change. *Medulla*: Injection of grey matter.

TABLE IX—*continued.*

B.—*Rabbits Immunised per os.*

Rabbit.	Weight.	Test-dose.	Result.
No. 111.	1640 grs.	0·7 c.c. "soluble" toxin intravenously.	No symptoms. Survived.
No. 112.	1440 grs.	Ditto.	Paralysis of hind legs on 2nd day. Died 3rd day. *Lung*: Congestion and hæmorrhage. *Liver*: Coccidiosis. *Small intestine*: Œdema, injection of submucosa. *Large intestine*: Slight œdema. *Spinal cord and medulla*: Degenerative change in nerve-cells, hæmorrhage.
No. 113.	1900 grs.	1·4 c.c. "soluble" toxin intravenously.	Paralysed on 2nd day. Died 3rd day. *Lung*: Congestion and hæmorrhages. *Spleen, liver and kidney*: Congestion. *Small intestine*: Injected. Œdema. *Large intestine*: Injection of submucosa. Œdema. *Appendix*: Injected. *Spinal cord*: Hæmorrhages in grey substance and degenerative change in nerve-cells. *Medulla*: As spinal cord.
No. 114.	1300 grs.	Ditto.	No symptoms. Survived. Killed and examined. *Lung, Liver and Kidney*: *Nil.* *Spleen*: Congestion. Cells of germ centres swollen. *Small intestine*: Injection and œdema of tunica propria, some small capillary hæmorrhages. *Spinal cord and medulla*: *Nil.*
No. 115.	1760 grs.	20,000 × 10^6 washed bacilli killed at 80° C. injected intravenously.	No symptoms. Survived. Killed and examined. *Lung*: *Nil.* *Spleen*: Severe congestion of pulp. *Small intestine*: Injection of tunica propria. *Spinal cord and medulla*: *Nil.*
No. 116.	1760 grs.	Ditto.	No symptoms. Survived. Killed and examined. Similar changes to those found in Rabbit 115.
		C.—*Control Rabbits.*	
No. 129.	1060 grs.	0·7 c.c. "soluble" toxin intravenously.	Found dead on morning of 2nd day. *Lung*: Congestion and hæmorrhage. *Spleen*: Severe congestion. *Liver*: Congestion and hæmorrhage. *Pancreas*: Hæmorrhages. *Kidney*: Congestion. *Small intestine*: Injection and œdema of submucosa, with eosinophile infiltration. *Appendix*: Eosinophile infiltrate. *medulla*: Hæmorrhages. *Spinal cord*: Injection of grey matter.
No. 130.	1200 grs.	1·4 c.c. "soluble" toxin intravenously.	Found dead on morning of 2nd day. Post-mortem findings as in Rabbit 129.
No. 137.	1900 grs.	20,000 × 10^6 washed bacilli killed at 80° C. injected intravenously.	No symptoms. Survived. Killed and examined. *Lung, liver, kidney*: *Nil.* *Spleen*: Enlarged. *Small intestine*: Injected. *Large intestine*: *Nil.* *Spinal cord and medulla*: *Nil.*

The titre of agglutinins for *B. dysenteriæ* (Shiga) in the serum of each of these rabbits was estimated ten days after the third immunising dose of vaccine. The suspension employed was made from a 24-hour agar culture and contained 2000 × 10^6 bacilli per c.c. Results were read after 4 hours at 37° C. and 24 hours at room temperature.

TABLE X.

A.—Rabbits injected subcutaneously with carbolised vaccine.

Rabbit . . .	No. 102	No. 103	No. 104	No. 105	No. 106	No. 107
Agglutinin titre for *B. dysenteriæ* (Shiga)	1/80	1/160	1/160	1/320	1/320	1/320

B.—Rabbits immunised *per os*.

Rabbit . . .	No. 111	No. 112	No. 113	No. 114	No. 115	No. 116
Agglutinin titre for *B. dysenteriæ* (Shiga)	1/10	*Nil*	*Nil*	1/40	1/40	*Nil*

From the experiment given in Table IX it will be seen that a certain degree of immunity can be produced in rabbits to the readily soluble fraction of dysentery toxin (so-called exotoxin) by both methods of immunisation. As to the degree of immunity in the two series A. and B. there is little to choose. Certainly those rabbits which had received subcutaneous inoculations of a carbolised vaccine showed more extensive pathological changes in the intestine and hæmorrhages were more numerous, which fact might, at first sight, be taken as evidence of some local immunity in the intestinal wall produced by the ingestion of the heat-killed vaccine. A closer inspection of the evidence, however, reveals the fact that the rabbits in question, Nos. 104, 105 and 106, were all infected with coccidia, so that it was impossible to consider these hæmorrhages as necessarily due to the test dose of toxin. It was therefore impossible to demonstrate conclusively any local intestinal immunity. Those rabbits which died in both series A. and B. succumbed to the nerve toxin. The data given in Table X show that the subcutaneous administration of vaccine is productive of a higher antibody titre than the oral immunisation.

As the experiment above detailed did not yield conclusive data of comparative value, it was decided to repeat in the hope that a definite conclusion might be arrived at. Two series of rabbits, six animals in each series, were immunised as before, one lot by subcutaneous inoculation of a carbolised vaccine, the other by the oral administration of heat-killed bacilli. In this case total leucocyte counts were made daily on each rabbit, both during the immunisation and after the administration of the test dose of toxin, and the temperature of each animal was similarly recorded. The object of this was to obtain some idea of the influence of these two methods of immunisation on the rabbits, and also to obtain further information as to the comparative effect of the test dose of toxin on the animals of these two series.

It has been pointed out by Lüdke (1911) amongst others that a leucocytosis occurs after the injection of dysentery toxin. Space would not permit of the inclusion in this paper of these daily observations on the leucocytic count and body temperature. Generally speaking there was an increase in the total white count and a rise in temperature of 1°–3° C. following each immunising dose. These changes were more severe in those rabbits receiving the sub-

cutaneous inoculations; apparently the ingestion of the vaccine is productive of less general reaction. The observations made four hours after the inoculation of the test dose of soluble toxin intravenously showed a rise in temperature of 2–3° C. accompanied generally with a fall in the total leucocyte count. This leucopenia occurred in all six of the rabbits immunised *per os*, whereas two of the six animals which had been inoculated subcutaneously showed no fall in the leucocyte count, and in two others only a moderate degree of leucopenia resulted.

The figures bearing on this point are here annexed:

Orally Immunised Series: Total Leucocytes.

Before test dose intravenously	11,567	11,898	10,245	9,915	9,135
Four hours afterwards	1,718	5,783	2,223	4,296	1,718

Series immunised by the subcutaneous method.

Before test dose intravenously	9,915	8,758	11,567	9,414	8,265
Four hours afterwards	1,720	5,453	11,563	5,783	18,930

TABLE XI.

Series A.—Rabbits immunised *per os*. (Three doses of $\frac{1}{3}$ of a Roux bottle culture.)

Rabbit.	Test-dose.	Symptoms.	Result.
No. 138.	2·0 c.c. "soluble" dysentery toxin.	Diarrhœa. Paralysis of fore and hind legs.	Died, 2nd day.
No. 139.	Ditto.	No symptoms.	Survived.
No. 140.	,,	Diarrhœa.	Died, 2nd day.
No. 141.	,,	Diarrhœa. Paralysis of fore and hind legs.	Died, 4th day.
No. 142.	,,	Diarrhœa. Incontinence of urine. Paralysis.	Died, 3rd day.
No. 143.	,,	Diarrhœa. Paralysis of fore and hind legs. Incontinence of urine.	Died, 4th day.
Series B.—Rabbits immunised by subcutaneous inoculation. (Three doses 50, 100 and 100 × 10^6.)			
No. 144.	2·0 c.c. "soluble" dysentery toxin.	Paralysis of hind legs. No diarrhœa. Incontinence of urine.	Died, 4th day.
No. 145.	Ditto. ,,	Paralysis of fore and hind legs. No diarrhœa. Recovered 7th day.	Survived.
No. 146.	,,	Paralysis of fore and hind legs. Slight diarrhœa on 3rd day.	Survived.
No. 147.	,,	Paralysis of hind legs. Diarrhœa.	Died, 3rd day.
No. 148.	,,	Paralysis of hind legs. Diarrhœa.	Died, 4th day.
No. 149.	,,	No symptoms.	Survived.

Five control animals were inoculated with varying doses of the soluble toxin with the following results:

Rabbit.	Weight.	Dose of toxin.	Result.
No. 155.	1350 grs.	0·1 c.c.	No symptoms. Survived.
No. 154.	1400 grs.	0·5 c.c.	Paralysed. Died 2nd day.
No. 153.	1500 grs.	1·0 c.c.	No symptoms. Survived.
No. 156.	1390 grs.	2·0 c.c.	Died, 24 hours after inoculation.
No. 157.	1440 grs.	2·0 c.c.	Paralysis of hind quarters and diarrhœa for two days. Recovered.

In Table XI the results of this experiment are detailed, the intravenous test dose of the soluble toxin being fixed at 2 c.c. Without reference to the unimmunised controls, which at the same time received doses varying from 0·1 c.c. to 2 c.c. (*bis*), the results would indicate a considerable superiority of subcutaneous over oral immunisation, there being in the one series three survivals out of six, and in the other, one only. If, however, recovery without symptoms be taken as the criterion of complete immunity there would appear to be only one such in each series. As had happened in a previous experiment when attempt was made to determine the minimum lethal dose of the soluble toxin, aberrant results occurred with the control animals of this experiment also, and probably, on the whole, it would be safest to conclude from the evidence available that solid active immunity to the soluble dysentery toxin is difficult and capricious by whatever method it is sought. No clear-cut evidence was obtained of an immunity similar to that which was realised in previous work when the test dose consisted of living dysentery bacilli.

The action of this soluble dysentery toxin intravenously administered has some points of resemblance with that of the so-called "shock" toxins, against which it is difficult in small animals to secure protection by active immunisation with killed vaccines, however administered.

ANTI-BODY RESPONSE TO ORAL IMMUNISATION.

The anti-body response to the ingestion of a dysentery vaccine was also investigated, the agglutination titre for *B. dysenteriæ* (Shiga) of the serum of each rabbit of Series A. being determined one week after each dose of vaccine. The sera were collected and kept in the cold room and all tested together with the same suspension, which was made from a 24-hour agar culture and contained 2000×10^6 bacilli per c.c. The results are recorded in Table XII.

From Table XII it will be seen that the agglutination titre for *B. dysenteriæ* (Shiga) rises appreciably with successive ingestions of bacilli. This is of interest, because Besredka's findings were entirely opposed to these. In his experiments he noted the appearance of agglutinins in the serum following the first ingestion of bacilli, after which they gradually disappeared, *the subsequent immunising doses of the vaccine giving rise to no anti-body response.* He

cites this observation in support of his contention that oral immunisation has produced an increased *local* resistance in the intestinal mucosa.

TABLE XII.

Time of observation.	Rabbit—				
	No. 144.	No. 145.	No. 146.	No. 147.	No. 148.
One week after 1st dose.	—	1/80	1/10	1/20	1/20
One week after 2nd dose.	1/10	1/80	1/20	1/80	1/20
One week after 3rd dose.	1/40	1/320	1/40	1/80	1/160

DISCUSSION.

The main object of these experiments, viz. the determination of the degree of local immunity in the intestinal mucosa produced by the ingestion of dysentery bacilli, was not achieved. The failure arose from two causes. First of all, no strain of *B. dysenteriæ* (Shiga) was found which, when inoculated into rabbits, gave rise to intestinal lesions to the exclusion of other pathological changes, and secondly, all attempts to separate endotoxin from exotoxin, on the lines of Olitsky and Kligler's work, proved fruitless. Certainly a portion of the toxin is readily dissolved out of the bodies of the dysentery bacilli. It was only necessary to suspend an agar culture in normal saline and to remove the bacilli by centrifugalisation to obtain a highly toxic supernatant fluid. This soluble fraction, however, produced the same lesions in the rabbits as the whole bacilli—changes not wholly confined to the central nervous system—and when heated for one hour at 80° C. all toxicity was lost. It must be admitted that the bacilli, even after washing ten times, still retained some of their toxicity. But when bacilli so treated were submitted to a temperature of 80° C. for one hour their toxicity was practically destroyed. Out of six rabbits inoculated with washed and heated bacilli, only one died—the one which received $20{,}000 \times 10^6$ bacilli—and though this animal showed hæmorrhages in the intestinal mucosa and other viscera, the presence of a heavy coccidiosis infection rendered it doubtful whether these lesions were to be placed to the account of the dysentery toxin or not.

CONCLUSIONS.

(1) The toxin of *B. dysenteriæ* (Shiga) affects principally the central nervous system (medulla and spinal cord) in rabbits. At the same time it acts upon the capillary circulation generally with the production of congestion and hæmorrhage in the various viscera.

(2) Exposure to a temperature of 80° C. for one hour markedly reduces its toxicity, especially for the central nervous system.

(3) It has been impossible to effect a separation between exotoxin and so-called endotoxin on the lines of Olitsky and Kligler.

(4) Though there is some indication in the foregoing experiments that oral immunisation produces a local immunity in the intestinal wall, this point remains undecided.

(5) The oral administration of the Shiga vaccine gives rise to less general reaction than does its inoculation subcutaneously.

(6) The antibody titre of the serum of rabbits immunised *per os* is appreciably raised by repeated ingestions.

(7) Attempts to secure, by subcutaneous and oral administration of killed vaccines, a solid active immunity against the potent soluble toxin of *B. dysenteriæ* (Shiga) intravenously administered, have not been attended with the striking success realised in previous work when active immunity was sought to the live bacilli similarly administered. Consequently, comparative estimates of the value of oral and subcutaneous methods in this connection are not at present possible.

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